Protein C Activators from Snake Venoms and Their Diagnostic Use

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Key Words

Protein C activators · Snake venom enzymes · Diagnostic tools

Abstract

Proteinases converting the zymogen protein C (PC) of vertebrates into activated PC have been detected in several snake venoms. Most PC activators have been purified from venom of snake species belonging to the genera of the *Agkistrodon* complex. Unlike the physiological, thrombin-catalyzed PC activation reaction which requires thrombomodulin as a cofactor, most snake venom activators directly convert the zymogen PC into the catalytically active form which can easily be determined by means of coagulation or chromogenic substrate techniques. Due to this feature, the fast-acting PC activator Pro-tac® from *Agkistrodon contortrix contortrix* (southern copperhead snake) venom has

found a broad application in diagnostic practice for the determination of disorders in the PC pathway. Recently, screening assays for the PC pathway have been introduced, based on the observation that the PC pathway is probably the most important physiological barrier against thrombosis.

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The Protein C Pathway

The protein C (PC) pathway belongs to the most important anticoagulant mechanisms of blood coagulation.

In the blood coagulation cascade, formation of thrombin – a serine proteinase with pronounced procoagulant properties – occurs in the final phase.

The plasma concentration of the enzymatically inactive prothrombin is approximately 100 times higher than would be necessary for

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complete blood coagulation – after total conversion into active thrombin. The healthy organism has the particular property of inhibiting the clotting system with an efficient negative regulation. Thereby, lesions to blood vessels are repaired by the controlled, locally limited formation of a blood clot. Several mechanisms that inhibit the propagation of the clotting process on the endothelium surface have been described. The anticoagulant PC pathway, one of the anticoagulation mechanisms, regulates blood coagulation by inactivating factors VIIIa and Va, and increasing the fibrinolytic activity [1, 2]. The mechanism can be described as follows: after binding of thrombin to thrombomodulin – a proteoglycan bound to the endothelium membrane – PC is activated [3]. Activated PC (APC) together with its cofactor protein S (PS) [4] inactivates factors VIIIa and Va by proteolytic degradation and therefore thrombin formation is reduced.

PC is a vitamin-K-dependent glycoprotein with a molecular weight of 62,000. In blood, it circulates as an inactive zymogen of a serine proteinase. PC is synthesized in the liver. Its half-life amounts to 8 h and its concentration in plasma is 4 µg/ml.

PS is also a vitamin-K-dependent blood clotting factor with a molecular weight of 70,000. Unlike other vitamin-K-dependent blood clotting factors, PS is not a zymogen of a serine proteinase; it is synthesized in the liver, in endothelial cells and in megakaryocytes. Its half-life amounts to 42 h and it circulates in blood at a concentration of 25 µg/ml. The cofactor activity of PS towards APC is regulated by the capacity of PS to bind to C4b-binding protein (C4bBP) [5]. C4bBP plays an important part in the complement system. The complex C4bBP PS has no APC cofactor activity. Approximately 60% of PS is bound to C4bBP.

PC Disorders

Two types of congenital heterozygous PC deficiencies can be distinguished: PC deficiency type I, where activity and antigen are equally reduced, and PC deficiency type II where clotting activity is lower than antigen concentration. In rare cases of homozygous PC deficiency, fulminant, fatal thromboembolism already occurs in the neonatal phase [6].

Acquired PC deficiency is relatively frequent, as PC belongs to the first factors that are reduced due to liver dysfunction. Disturbances of PC synthesis are mainly observed in case of hepatic diseases, vitamin K deficiency and coumarin therapy. Increased conversion of PC takes place above all in disseminated intravascular coagulation, postoperatively, in insulin-dependent diabetes mellitus and in renal failure.

Increased PC concentration may be due to drugs, e.g., ovulation inhibitors or anabolics. Increased PC levels are also found in pregnancy.

Concentrations between 65 and 150% correspond to the normal range. Concentrations between 20 and 70% imply an increased risk of thromboembolism.

PS Disorders

Three types of familiar, heterozygous PS deficiencies can be distinguished. In type I, total and free PS antigen level are reduced; in type II, PS activity is reduced, but the antigen level of free and total PS is normal; in type III, PS activity and free PS are reduced, but the total PS antigen remains normal [7, 8]. Homozygous PS deficiency is extremely rare.

Acquired PS deficiency is mainly observed in case of hepatic diseases, vitamin K deficiency, coumarin therapy, chronic inflamma-

tory intestinal diseases, disseminated intravascular coagulation and nephrotic syndrome.

Concentrations between 60 and 160% correspond to the normal range, concentrations below 60% bring about an increased risk of thromboembolism.

APC Resistance

APC resistance is associated with the most common hereditary defect predisposing to venous thromboembolism [9]. More than 97% of APC resistance cases are caused by a point mutation in the factor V gene, replacing Arg 506 by Gln in the resulting factor V protein, also referred to as factor V Leiden [10]. Heterozygotes for this mutation have a 5- to 10-fold increased risk of venous thrombosis, while homozygotes are even 50–100 times more susceptible. Due to the high prevalence of factor V Leiden in the normal population [11], this factor should be the first parameter to be evaluated in any scheme assessing a hereditary risk.

PC Activators

The following snake venoms have been described to contain PC activators [12–17]: *Agkistrodon contortrix contortrix*, *Agkistrodon contortrix mokasen*, *Agkistrodon contortrix pictigaster*, *Agkistrodon piscivorus piscivorus*, *Agkistrodon piscivorus leucostoma*, *Agkistrodon bilineatus*, *Agkistrodon blomhoffii ussuriensis*, *Agkistrodon halys halys*, *Bothrops moojeni*, *Bothrops pradoi*, *Vipera lebetina*, *Daboia russelli*, *Cerastes cerastes*, *Trimeresurus mucrosquamatus*.

First results on the isolation of thrombomodulin-independent and fast-activating PC activator from *A. contortrix contortrix* (south-

ern copperhead snake), and related PC-activating properties of enzymes from venom of *Agkistrodon* subspecies and in venom of *A. piscivorus* (eastern cottonmouth snake), *Agkistrodon bilineatus* (tropical moccasin snake), *B. moojeni*, *B. pradoi* (Prado's pit viper), *Vipera lebetina* (desert adder) and *Cerastes cerastes* (Egyptian sand viper) were presented in 1985 at the Symposium on Animal Venoms and Hemostasis in San Diego, Calif. [18].

The commercial preparation Protac® – the purified form of the fast-acting PC activator from *A. contortrix contortrix* – has considerably simplified the determination of PC [19–21]. Protac converts the zymogen PC very quickly, independently of thrombomodulin, into a serine proteinase which exerts the enzymatic and anticoagulant effects of PC. In contrast to thrombin, the activation product of Protac is not influenced by the inhibitory effect of PC inhibitor. Consequently, time-consuming adsorption steps to separate PC from its inhibitor for the determination of PC are no longer required. Protac also activates PC from cattle, sheep, goats, rabbits, horses and pigs.

Properties and Mode of Action of PC Activators

Protein C activators are mainly characterized from *Agkistrodon* snake venom. According to a comparative study, PC activators can be divided into two groups [16]:

PC Activators from New World Species of the Genus Agkistrodon

Protein C Activators from A. contortrix Venom. *A. contortrix contortrix* crude venom is composed of up to 80 different proteins [22, 23]. Characterizations made by different laboratories [24–28] revealed the following prop-

erties for the PC activator from *A. contortrix contortrix*. The activator is a serine proteinase with a molecular weight of 36,000–40,000 D and shows an isoelectric point in the pH range of 7.5–9. PC is activated by limited proteolysis and splits off the substrate Tos-Gly-Pro-Arg-pNA. The anticoagulant and amidolytic activity is inhibited by serine proteinase inhibitors such as NPGB, PMSF, DFP, H-D-Phe-Pro-Arg-chloromethylketone and the active center acylating inhibitor for serine proteinases, 2-ethoxy-6.7-dimethoxy-4H-3.1-benzoxazine-4-one [29]. The amino acid sequence of the PC activator from *A. contortrix* has been completely elucidated [30]. The protein is composed of 231 amino acids with a calculated molecular weight of 25,095 D. Similarly to mammalian serine proteinases, the PC activator has His-40, Asp-85 and Ser-177 in the catalytic active center. The primary structure of the PC activator from *A. contortrix contortrix* shows many homologies to other snake venom enzyme proteinases such as batroxobin from *Bothrops atrox*, flavoxobin from *Trimeresurus flavoviridis*, gyroxin from *Crotalus durissus terrificus*, crotalase from *C. adamanteus* and cerastobin from *Cerastes vipera*.

Protein C Activator from A. bilineatus. The PC activator from *A. bilineatus* [31] has also been characterized. This activator has been shown to have a molecular weight of 35,000–38,000 D by SDS-PAGE under reducing and nonreducing conditions. Analysis of the amino acid sequence revealed a strong homology to PC activator from *A. contortrix* venom.

Protein C Activators from Old World Species of the Genus Agkistrodon

Protein C Activator from A. blomhoffii. A PC activator with a molecular weight of 44,000 D has been determined by SDS-PAGE in *A. blomhoffii ussuriensis* (Ussuri mamushi) venom [15]. This PC activator was described

to prolong the aPTT in human plasma, and after intravenous injection to rats the aPTT proved to be prolonged, plasminogen activity increased and FV procoagulant activity decreased.

Protein C Activator from Agkistrodon halys halys. A PC activator with a molecular weight of 36,000–40,000 D and an antithrombotic effect in rats [14, 32] has been isolated from *A. halys halys* (Asian pit viper) venom. The difference between PC activators from New and Old World snake venom consists in the fact that PC activators from *Agkistrodon* from the New World have a molecular weight of less than 40,000 D and a pH optimum of 7.0–8.5, and activate PC from rat plasma to a lesser extent than PC from human plasma. PC activators from *Agkistrodon* species from the Old World have molecular weights greater than 40,000 D and a pH optimum between 5.0 and 6.5, and activate PC from rat plasma to a higher extent than PC from human plasma.

PC Assays Using a PC Activator from Snake Venom

The presently applied methods of PC determination are either functional or immunochemical. Functional assays are either clotting assays or based on determinations using chromogenic assays [19, 33, 34]. They have been largely simplified by the introduction of Protac. The chromogenic assay is performed as follows: PC in plasma is activated with Protac and APC splits off a chromogenic substrate. This test is very easy and specific, can easily be automated for clinical use and shows an extraordinary accuracy.

In the PC clotting assay, patient plasma is prediluted with PC-deficient plasma to exclude any disturbing factors. PC is activated with Protac and the inhibitory effect of APC is determined by aPTT. Prolongation of the

clotting time is proportional to the activity of PC in patient plasma. This assay too can be automated.

PS Assays Using a PC Activator from Snake Venom

Basically three types of assays can be recognized [35–40]: (1) Immunological assays for total PS; (2) Immunological assays for free PS; (3) PS functional assays. The APC cofactor activity can be measured as the prolongation of a prothrombin-time-based clotting assay [41], in which diluted test plasma, PS-depleted plasma containing Protac-activated PC, bovine brain tissue thromboplastin and calcium ions are mixed. The clotting time prolongation is proportional to the PS content in patient plasma. Chromogenic methods and aPTT methods have also been described.

Screening Assays for the PC Pathway

Recently, screening assays for the PC pathway have been introduced which are based on the observation that the PC pathway is probably the most important physiological barrier against thrombosis. For this reason, identification of a dysfunctional PC pathway should be the first step in order to assess an individual thrombosis risk. Several groups have described similar functional assays which are based on the following principle [42]: a screening test, e.g. aPTT, is performed on the sample with or without previous or simultaneous activation of PC by Protac.

The two clotting times are required in order to compensate any changes of coagulation factors which influence the individual aPTT of a sample. A functional PC pathway leads to a massive prolongation of the clotting

time in the sample with the activator and thus to a PC ratio (the ratio of the clotting times with and without Protac) above the cut-off value for normals. The PC ratio is slightly instrument dependent and can be normalized with the help of a calibration plasma.

Pathological results are found in almost all patients with factor V Leiden or with PC deficiency, whereas the detection of PS dysfunction is less efficient. Other factors which influence the assay are lupus anticoagulants, the prothrombin mutation 20210 G/A or elevated factor VIII.

APC Resistance Assay

The following example of APC resistance assay (APC-R assay) was performed with the PC activator from *A. contortrix contortrix* [unpubl. data].

Sample plasma is prediluted with factor-VV-deficient plasma and incubated at 37°C with a specific factor V activator from *Daboia russelli* and phospholipids in the presence and absence of Protac. Coagulation is triggered by the addition of a factor-V-dependent prothrombin activator from *Notechis scutatus* in the absence of calcium. The time until clot formation is recorded. The ratio of clotting time with/without added Protac is calculated. Homozygous factor V Leiden gives a ratio below 1.0, heterozygous factor V, a ratio between 1.0 and 2.0, and wild-type, a ratio above 2.0.

Conclusions

The active component Protac is a protease which causes the activation of PC and is used for the determination of PC, PS, APC-R and in global assays for determination of PC pathway disorders. Properties of PC activators

from snake venom have been determined in a relatively short time and the PC activator from *A. contortrix contortrix* is now widely used as a diagnostic tool in medical research and clinical use.

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