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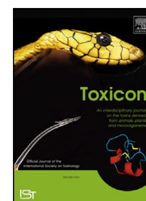

Agkihpin, a novel SVTLE from *Gloydius halys* Pallas, promotes platelet aggregation in vitro and inhibits thrombus formation in vivo in murine models of thrombosis

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A B S T R A C T

In previous work, a snake venom arginine esterase (SVAE), agkihpin from the venom of *Gloydius halys* Pallas, was isolated and its biochemical data including Mr, PI, amino acid components and sugar content was collected. Here, the agkihpin was cloned and further characterized and we found that agkihpin could promote ADP-induced platelets aggregation, hydrolyze fibrin, cleave Aα and Bβ chains of fibrinogen and reduce the thrombosis induced by thrombin. Moreover, agkihpin hydrolyzed TAME with optimum temperatures at 30 °C–45 °C, and the hydrolysis was inhibited by EDTA, PMSF, DTT and promoted by Ca^{2+}, Fe^{3+}, Mg^{2+}, Zn^{2+}. The sequence features of agkihpin were detected as follows: the N-terminal residues was determined as I(V)L(Y)GDDECNINE by protein sequencing; the ORF was determined as 705 bp, and the deduced amino acid sequence was identified by peptide mass fingerprinting; the cysteines, cleavage sites, active sites and substrate binding sites of snake venom thrombin-like enzyme (SVTLE), were all conserved in amino acid sequence of agkihpin; 2 Leu(Tyr), 4 Asn and 121 Ile in amino acid sequence of agkihpin were first found in the amino acid sequences of SVTLEs. These findings indicated that agkihpin is a novel SVTLE. What's more, due to its several advantages of fibrino(gen)olytic and thrombosis-reduced activities, and devoid of bleeding risk, agkihpin may be developed into a thrombolytic drug in the future.

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1. Introduction

Some snake venom arginine esterase (SVAE) preferentially released either fibrinopeptide A or B from fibrinogen to produce abnormal fibrin clots composed of short polymers, which were rapidly dispersed and no longer cross-linked by activated factor XIII, resulting in the disruption of the blood coagulation system of victims (Zhang et al., 2007), so SVAE was often referred to as snake venom thrombin-like enzyme (SVTLE). In current, SVAE in drug research was mainly focused on the anti-coagulation (Savchik et al., 2013), hemostasis (Vivas-Ruiz et al., 2013), analgesia (Bladen, 2013), anti-thrombus (Lei et al., 2014) and anticancer (Liu et al., 2014).

Previously, dozens of snake venom proteins had been purified and applied for clinical treatment and diagnosis (Morais et al., 2013; Koh and Kini, 2012). For example, ahylsantinfarctase and Chinese herbal medicine were injected jointly to treat diabetic foot ulcer and gangrene (Wang, 2002); Captopril played the antihypertensive and antihyperlipidemic roles on hypertensive patient (Geleta et al., 2016), as well as diagnostic role in subjects with high aldosterone-to-renin ratios (Kim et al., 2016); Enalapril was utilized to therapy heart failure (Vilela-Martin, 2016); In addition, Tirofiban and fondaparinux (either separately or combined) were used to treat post-percutaneous coronary intervention (PCI) patients with acute coronary syndrome (ACS) and concurrent renal insufficiency. However, some of the existing snake venom protein drugs may have some side effects, such as severe thrombocytopenia (TIT) (Ibrahim et al., 2016), and bleeding during circulating thrombolysis (Zhang et al., 2013). Therefore, it is a challenging task to discover some new snake venom protein that with high pharmacological effect and small side effects.

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In our previous research, agkihpin, a SVAE, was isolated from the venom of *Gloydius halys* Pallas, that with molecular mass of 25.46 KDa, isoelectric point of 7.43 and protein component of 235 amino acid residues (Hu and Shu, 2006). In this study, we further investigated the cloning and characterization of agkihpin, and aimed to supply more experimental evidences and information for pharmacological effect of agkihpin.

2. Materials and methods

2.1. Materials and animal

Living *G. halys* Pallas snakes were collected in the area of Zhejiang, an east province of China. Lyophilized crude venom of *G. halys* Pallas was the stock of our laboratory. Agkihpin was prepared through the procedure as we described previously (Hu and Shu, 2006). Sephadex G-75 gel (superfine) and Sephrose CL-6B were the products of Amersham Bioscieces of GE-Healthcare. Total RNA isolation kit and first-strand cDNA synthesis kit were purchased from TIANGEN BIOTECH Co., LTD. LA-Taq DNA polymerase and DNA fragment extraction kit were purchased from TaKaRa Biotechnology Co. Ltd. pUCm-T vector was purchased from Bio Basic Inc. Antibodies for PAR1, PAR4 and GAPDH, and peroxidase coupled secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. All other reagents used were analytical grade. Kunming mice (certificate number SCXK Gui 2009 0002) and SD rats (certificate number SCXK 2007-0005) were obtained from Guangxi Experimental Animal Center (Nanning, China). Prior to the test, animals were raised with granulated feed and water free. The clean conditions included a room temperature of 23.0 ± 2 °C, humidity of 56%, 12:12 h interrupted lighting, and 24 h regular UV disinfection and ventilation without strong light and sound stimulation.

2.2. Activity and mechanism assay of agkihpin on platelets aggregation

For platelet functional assays, blood was collected from a healthy donor who had not received any medication for at least 2 weeks. The blood was dispensed into a tube containing 0.15 mol/L sodium citrate (9:1 v/v), an anticoagulant. The whole blood was centrifuged at 1000 rpm for 10 min to obtain platelet-rich plasma (PRP). And the platelet-poor plasma (PPP) was prepared from the remainder by centrifuging at 3000 rpm for 10 min. PRP was diluted to 2.50×10^{11} platelets/L using PPP (Jangprase and Rojnuckarin, 2014). Platelet aggregation was measured photometrically by using an aggregometer (Payton, module 600B, Canada) under continuous 900 rpm stirring at 37 °C. 400 μl diluted PRP was incubated with different amounts of agkihpin for 5 min before adding the agonist (ADP) to a final concentration of 5 mM (Tsai et al., 2012). After the photometrical measure, the sample would be used for the Western blots detection of protease-associated receptors (PARs) expression in platelets according to the method described by Schlagenhauf et al. (2012).

2.3. Fibrinolytic activity and fibrinogen degradation assays of agkihpin

Fibrinolytic activity analysis was conducted by using a fibrin plate method (Peichoto et al., 2007). Fibrin plates were prepared by the addition with 5 units of thrombin to a 10 ml preheated 50 mmol/L Tris-HCl buffer solution, which contains 2% agarose, 0.4% bovine fibrinogen and 0.18 mol/L NaCl, pH 7.5. A fibrin clot was formed on a level surface. 10 μl sample solution (containing PBS, 0.6 U urokinase, 15 μg crude venom and 15 μg, agkihpin, respectively) was placed into holes on a fibrin plate and incubated at 37 °C for 18 h, then the areas of clearance were analyzed (Wang et al., 2014). Fibrinogenolytic activity was assayed by incubating 0.1 ml of 0.2% bovine fibrinogen solution with 0.05 ml agkihpin solution (containing 2 μg) in 50 mM Tris-HCl buffer (pH 7.5) at 37 °C for 0.5–16 h (Hung et al., 1994). Samples were subsequently analyzed by SDS-PAGE.

2.4. Effects of temperature, cations and inhibitor on AEase activity of agkihpin

The effects of temperature, cations and inhibitors on agkihpin activity were tested with the TAME assay described in previous research (Hu and Shu, 2006). Optimum temperature for agkihpin (21 μg/ml) was determined by measuring activity at optimum pH 8.2 and at 10°C-70 °C for 90 min. The effect of 5 mmol/L $MgCl_2$, $CaCl_2$, $ZnCl_2$, EDTA, $FeCl_3$, β-mercaptoethanol (BME), DL-Dithiothreitol (DDT) or Phenylmethanesulfonyl fluoride (PMSF) on agkihpin activity was investigated by assaying the enzyme in the presence of these compounds (Zhang et al., 2010).

2.5. Influence of agkihpin on thrombin-induced venous thrombosis in the inferior vena cava of rats

Thrombus formation was induced by using the method described by Hladovec (Hladovec, 1986). Briefly, 30 SD rats (300 ± 30 g) with half males and half females were randomly divided into five groups ($n = 6$): a negative control group (same volume of normal saline (NS)), three agkihpin groups (160, 320 or 640 μg/kg, respectively) and a tissue plasminogen activator (tPA) group (160 μg/kg). Different doses of agkihpin, tPA and NS were injected by the tail veins of the rats. Sodium pentobarbital (35 mg/kg) was intraperitoneally injected as an anesthetic after 1 h. The rats were placed in a dorsal position and the abdominal wall was longitudinally opened to move the internal organs and expose the inferior vena cava and its branches (the left and right branches were ligated). Thrombin (10 IU/kg) was injected into the femoral vein to induce thrombosis, the line on the margin of the left renal vein was immediately ligated after injection for 20 s, and the abdominal cavity was temporarily closed. After 10 min, the downside line was ligated, the inferior vena cava segment between the two ligatures was opened to rapidly measure thrombosis, and the remaining blood was removed with the filter paper. The length and dry weight of the thrombus were measured. The thrombus weight (mg) was equal to the dry weight of the thrombus divided by the length of thrombus, and then divided by the body weight of rats (Zhang et al., 2013). The protocol for this study was approved by the Animal Care and Use Committee of Guangxi Medical University.

2.6. Bleeding test of agkihpin on mice

To evaluate the bleeding risk of agkihpin, a mouse subcutaneous bleeding study was carried out. Thirty Kunming mice were randomly divided into six groups, and five groups of mice were injected subcutaneously with NS, crude venom (positive control, 1 μg/g weight of rat) from *G. halys*, and a series dosage of agkihpin (4 μg/g, 16 μg/g and 32 μg/g weight of mouse) on the back of mice respectively. Mice were sacrifice and their back skins were peel off after 24 h of injection, and the hemorrhagic area around 12.5 mm of pinhole of the skin were measured and recorded (Perzborn et al., 2005). The smallest unit of bleeding activity was defined as the smallest dosage that causing 10 mm^2 hemorrhagic area. The protocol for this study was approved by the Animal Care and Use Committee of Guangxi Medical University.

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