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Aegyptin inhibits collagen-induced coagulation activation *in vitro* and thromboembolism *in vivo*

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ABSTRACT

Aegyptin is a mosquito salivary gland protein and potent inhibitor of platelet aggregation. Aegyptin binds to the von Willebrand factor-binding site on collagen and prevents its interaction with platelets. Because collagen also induces plasma clotting by activation of factor XII, we evaluated the effects of aegyptin on collagen-induced coagulation activation and how it interferes with thrombosis in three different *in vivo* models. Our results demonstrate that aegyptin abolishes collagen-induced clot formation and thrombin generation in platelet-free plasma. Aegyptin has no antithrombotic activity in the arteriovenous shunt model (collagen-independent) but it prevents laser-induced collagen-mediated thrombus formation in rats. Furthermore, aegyptin protects mice from collagen and epinephrine-induced thromboembolism. Therefore, aegyptin has a dual antithrombotic mechanism: inhibition of platelet-collagen interaction and collagen's pro-coagulant activity. This is the first description of a collagen-binding protein that also inhibits collagen-mediated coagulant activity.

1. Introduction

Damage to the vascular endothelium often results in collagen exposure to the blood which plays an important role in the initial stages of hemostatic plug formation upon vascular injury. The interaction of circulating platelets with collagen is a multi-stage process that involves several receptors that initiate cellular activation and platelet aggregation [1]. Collagen can directly interact with either glycoprotein receptors on the platelet surface including integrin α2β1 and glycoprotein VI (GPVI), or indirectly via binding of von Willebrand factor (vWF) to the platelet surface through the glycoprotein Ib/V/IX complex [2].

In addition to its well-established role in primary hemostasis, i.e., platelet adhesion and aggregation, collagen was recently shown to initiate blood coagulation through the intrinsic pathway [3]. *In vitro*, collagen interacts with factor XII, enabling its activation and leading to subsequent thrombin generation. Therefore it has been proposed that there exists a dual role for collagen in thrombus formation [4].

Platelet inhibitors, such as aspirin and ADP receptor antagonists, or anticoagulants such as heparin, pentasaccharide and vitamin K antagonists are the current mainstay of treatment and prevention of thrombosis [5–6]. Although these drugs reportedly improve survival in patients with ischemic disorders, these drugs also have limitations and side effects such as excessive bleeding. Therefore, identifying novel drugs capable of preventing thrombus formation without increasing the risk of hemorrhage has been the focus of experimental and clinical investigations. In this context, it has been proposed that targeting the intrinsic pathway of coagulation would be advantageous for treating thromboembolic diseases [7–8].

Salivary glands from blood-sucking animals are a rich source of bioactive molecules that counteract hemostasis [9–11]. These molecules have been regarded as important tools for the study of the hemostatic process, and have provided a valuable source of chemical prototypes to develop antithrombotic compounds [12–13]. Aegyptin is a 30 kDa mosquito salivary gland protein that binds to collagen ($K_D \approx 6.0$ nM) and inhibits platelet aggregation. Aegyptin attenuates platelet adhesion to either soluble or fibrillar collagen and inhibits vWF interaction with collagen under static and high-shear conditions [14]. Surface plasmon resonance identified a high-affinity interaction between RGQOGVMGF (where O is hydroxyproline), a peptide corresponding to the collagen-binding site for vWF, and aegyptin [15]. Aegyptin also recognizes the peptides (GPO)(10) and GFOGER with low affinity (micromolar range), which represent the glycoprotein VI- and integrin alpha2beta1-binding sites on collagen, respectively [15].

In the present study, we evaluated the effects of aegyptin on blood clotting induced by collagen and tested its effect on different models of induced thrombosis. Our results showed that aegyptin

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inhibits the collagen-mediated acceleration of human plasma clotting which may contribute to its effective antithrombotic activity *in vivo*. Our data support a dual mechanism of action in which aegyptin targets both the pro-aggregatory and the procoagulant activities of collagen. This molecule might be regarded as an important tool to dissect the hemostatic role of collagen *in vivo* as well as a potential agent for the treatment of human cardiovascular diseases.

2. Material and methods

2.1. Reagents

Recombinant aegyptin was produced in 293-F cells, purified and quantified as previously described [14]. Standard collagen (equine fibrillar type I Horm, type I/H) was purchased from Chrono-Log Corp. (Haverstown, PA, USA). The chromogenic substrate for thrombin (H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroaniline dihydrochloride, S-2238) was purchased from Diapharma (Westchester, OH, USA). Gly-Pro-Arg-Pro amide (GPRP), L-α-phosphatidylcholine (PC) and L-α-phosphatidylserine (PS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phospholipid vesicles (PC/PS) composed of 75% PC/25% PS (w/w) were prepared by sonication. Briefly, phospholipids in chloroform were dried with a stream of N2 and lyophilized. Lipids were resuspended in 50 mM Tris–HCl and 150 mM NaCl (pH 7.5) sonicated for 10 min and adjusted to a final concentration of 500 μM. Anasedan (xylazin) and Dopalen (ketamin) were purchased from Agribrands (Rio de Janeiro, Brazil).

2.2. Animals

Male and female Wistar rats and Balb/c mice were housed under controlled conditions of temperature (24 ± 1 °C) and light (12 h light starting at 7:00 a.m.), and all experiments were conducted in accordance with standards of animal care defined by the Institutional Committee (Institute of Medical Biochemistry, Federal University of Rio de Janeiro).

2.3. Plasma clotting assays

The effect of aegyptin on plasma clotting induced by collagen was evaluated on an Amelung KC4A coagulometer (Labcon, Heppenheim, Germany) as previously described with slight modifications [3]. The coagulation time was monitored in platelet-rich plasma (PRP) or in platelet-poor plasma (PPP) supplemented with 10 μM PC/PS. Human blood samples were collected from healthy donors in 3.8% trisodium citrate (9:1, v/v). PRP was obtained by centrifugation at 800*g* for 10 min, and PPP was obtained by further centrifugation at 2000*g* for 10 min.

Briefly, 1 μM recombinant aegyptin and 50 μL collagen type I were incubated for 10 min at 37 °C before adding 50 μL of either PRP or PPP supplemented with 10 μM PC/PS. After 10 min, clotting was triggered by addition of $CaCl_2$ (final concentration 5 mM).

2.4. Measurement of thrombin generation in plasma

Thrombin generation was continuously measured in PPP supplemented with 10 μM PC/PS, as previously described [11]. Briefly, 50 μL PPP, 50 μL either TBS or aegyptin, and 50 μL collagen type I (50 mg/mL) were incubated for 10 min at 37 °C. The reaction was initiated by the addition of $CaCl_2$ (final concentration 5 mM), and 10 μL aliquots were transferred every 10 s into microplate wells containing 40 μL of TBS-EDTA buffer. To impair plasma clotting, reactions were carried out in the presence of 10 mM GPRP. Thrombin activity was determined by the addition of 200 μM of the chromogenic substrate S-2238. Hydrolysis of the substrate was detected using a Versamax Microplate Reader (Molecular Devices, Menlo Park, CA, USA), equipped with a microplate mixer and heating system. Reactions were recorded continuously at 405 nm, for 20 min, at 37 °C. The total volume of the reactions was 100 μL.

2.5. Thrombus formation in the arterio-venous shunt model

Antithrombotic activity in an extracorporeal shunt was investigated as previously described [16]. Rats (both sexes, 200–250 g body weight) were anesthetized with an intramuscular injection of xylazine and ketamine (16 and 100 mg/kg, respectively). Through a longitudinal incision in the skin over the trachea along the midline, the left jugular vein and the right carotid artery were cautiously freed from the surrounding tissue. The right carotid artery and the left jugular vein were cannulated, and a solution of aegyptin was injected into the artery and allowed to circulate for 15 min before initiating the surgery for thrombosis induction.

Concurrently, an extracorporeal shunt was prepared. This shunt consisted of two 6-cm pieces of polyethylene tubing (1.3 mm inner diameter) with both ends cut at an angle; one end of each piece of tubing was forced into the end of a central, 6-cm tube (1.6 mm inner diameter) with a 5 cm length of silk thread drawn through it to leave 1.0 cm outside the tubing at one end and 4.0 cm inside the tubing. This shunt was filled with a heparin solution (50 U/mL). One end of the shunt was inserted into the left jugular vein, and the other end was subsequently inserted into the right carotid artery to establish blood circulation. After 20 min of blood circulation through the shunt, the blood flow was stopped at the arterial side of the tubing with a pinch-clamp and the center of the extracorporeal shunt was isolated. From this segment, the silk thread coated with the thrombus was carefully pulled out with the assistance of the piece of thread that remained external to the shunt. The wet thrombus weight was immediately determined by subtracting the weight of the wet thread.

2.6. Photochemically induced carotid artery thrombosis in rats

The effect of aegyptin on arterial thrombosis was evaluated in an established rat model [17]. Animals were anesthetized as described above. The right common carotid artery was isolated through a midline cervical incision, and the blood flow was monitored continuously using a 1PR Doppler flow probe and a TS420 Flowmeter (Transonic Systems, Ithaca, NY). Rose bengal dye (90 mg/kg body weight) was diluted to 60 mg/mL in PBS and administered via slow injection (over 2 min) through the cava vein. Just before injection, the common carotid artery was transilluminated with a 1.5-mV, 540 nm green laser (#25-LGR-193–249, Melles Griot Inc., Carlsbad, CA) immediately proximal to the Doppler probe from a distance of 3 cm. The mean carotid artery blood flow was monitored until stable occlusion occurred (defined as a blood flow of 0 ml/min for $\geqslant$5 min), at which time the experiment was terminated. Either aegyptin or PBS (control) was injected into the cava vein 15 min before initiating the Rose bengal administration.

2.7. Collagen/epinephrine-induced pulmonary thromboembolism

Balb/c mice were anesthetized with intramuscular xylazin (16 mg/kg) followed by ketamine (100 mg/kg). Either aegyptin (0.5 and 1.0 mg/kg) or vehicle was slowly injected into the inferior vena cava 5 min prior to challenge. A mixture of 0.8 mg/kg collagen and 60 μg/kg epinephrine was then injected into the inferior vena cava. Animals still alive after 30 min were considered to be survivors.

2.8. Statistical analysis

Results were expressed as the mean ± standard error. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). One-way analysis of variance (ANOVA) complemented by the Dunnett post hoc test was used for comparisons between test groups. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Aegyptin abolishes collagen-mediated acceleration of human plasma clotting

It was recently demonstrated that collagen accelerates plasma clotting by activating the intrinsic pathway [3]. Remarkably, this effect was observed regardless of whether phospholipids or platelets are present as procoagulant lipid surface [3]. To investigate the possible effect of aegyptin on inhibiting the procoagulant effect of collagen, plasma coagulation experiments were performed using either platelet poor plasma (PPP) supplemented with PC/PS vesicles or platelet rich plasma (PRP). As previously reported [3], collagen significantly accelerates plasma clotting in both PPP and PRP (Fig. 1A and B, respectively). Aegyptin completely blocked collagen-induced coagulation in both experimental conditions but showed a more pronounced effect in the absence of platelets. In fact, assays in the presence of 1 μM aegyptin showed a prolongation of plasma clotting by ~1.5-fold, compared to control values (451.7 ± 52.4 versus 307.3 ± 15.9 s). A possible explanation for this observation is the binding of calcium ions to collagen [18], which may direct interfere with time to achieve plasma clotting. This hypothesis is reinforced by the observation that aegyptin has no effect on plasma clotting in the absence of collagen (data not shown).

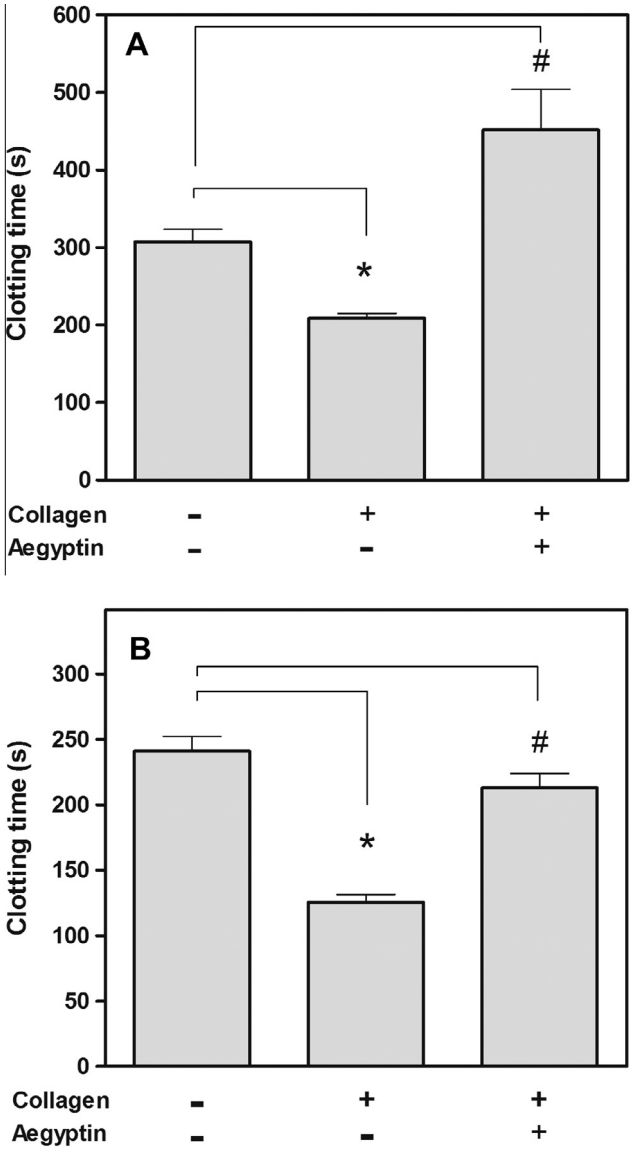

Fig. 1. Aegyptin abolishes collagen-mediated acceleration of human plasma clotting. Either platelet poor plasma supplemented with 10 μM PC/PS vesicles (A) or platelet-rich plasma (B) were pretreated with 1 μM aegyptin. Preparations were then incubated with either collagen type I (50 μg/mL) or vehicle solvent (control). Coagulation was initiated by the addition of $CaCl_2$ (5 mM final concentration). Values are represented as the mean ± SE; $n = 5–8$; $^{*}P < 0.05$ vs control; $^{\#}P < 0.05$ vs conditions with collagen.

3.2. Aegyptin delays collagen-induced thrombin generation in the plasma

Initiation of the intrinsic pathway by collagen results in an increase in thrombin generation in plasma [3]. Because aegyptin impaired collagen-induced coagulation, we evaluated its effect on collagen-mediated thrombin generation in PPP. Fig. 2 shows that addition of collagen to PPP accelerates thrombin formation and increases the amount of enzyme formed in the assay. This effect is completely blocked by the addition of recombinant aegyptin to the assay, which caused a delay in thrombin generation and a significant reduction in thrombin formation (Fig. 2).

3.3. Aegyptin displays effective anti-thrombotic activity in vivo

The *in vivo* antithrombotic activity of aegyptin was evaluated by employing three models of experimental thrombosis. We first used an arteriovenous shunt model in rats. This model is based on the extracorporeal circulation of blood and is independent of collagen exposure because it does not cause endothelial denudation [16]. Aegyptin was ineffective in counteracting thrombus formation in this model (Fig. 3), which supports its proposed mechanism of action.

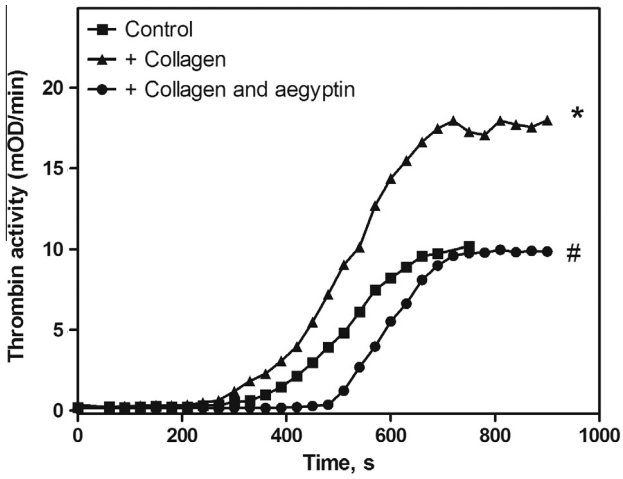

Fig. 2. Aegyptin inhibits thrombin generation in human plasma. Thrombin generation was measured by hydrolysis of S-2238 (0.2 mM) as described in the Materials and Methods section. Curves show thrombin formed in plasma (results expressed as Vmax, mOD/min) in either the absence or presence of collagen and aegyptin. Each point represents mean ± SE of three independent determinations. $^{*}P < 0.05$ vs control; $^{\#}P < 0.05$ vs conditions with collagen.

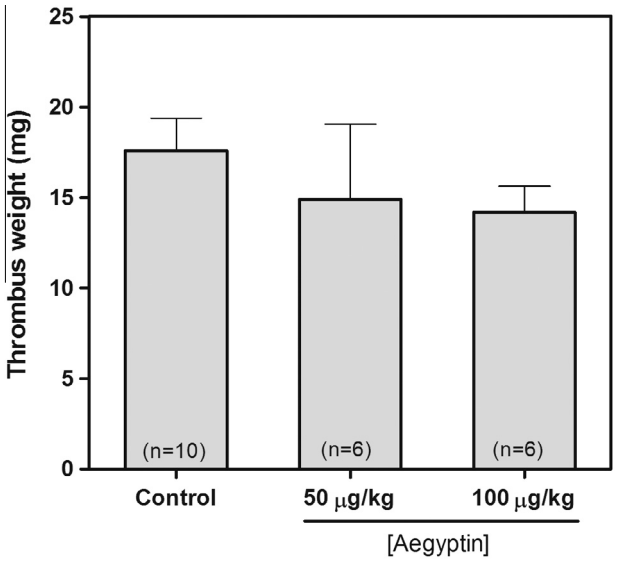

Fig. 3. Aegyptin shows no antithrombotic activity in an arteriovenous shunt model. Aegyptin (50 or 100 μg/kg) or PBS (control) was injected into the carotid artery 15 min before initiating the surgery to induce thrombosis. The shunt was assembled by connecting two cannulae with a slightly curved tube containing a 5 cm cotton thread and filled with heparin solution (50 U/mL). The extracorporeal circulation was maintained for 20 min, and the thrombus formed on the cotton thread was then removed from the shunt tube. Each bar represents the mean ± SE, and the number of animals tested for each condition is reported here.

Next, we examined the effects of aegyptin in a laser-induced carotid thrombus formation model. This model is based on vessel injury, and thrombus formation is dependent on collagen exposure, which leads to both platelet and fibrin accumulation [19]. In the presence of aegyptin, the amount of time to occlusion was significantly different between the control rats and rats treated with 50 μg/kg of the inhibitor (19.37 ± 2.38 vs 54.57 ± 9.44 min) (Fig. 4A). Interestingly, rats treated with 100 μg/kg of aegyptin were resistant to arterial occlusion, and occlusion did not take place before 60 min in all animals tested (Fig. 4A).

The efficacy of aegyptin in inhibiting thrombus formation was also measured in a murine model of lethal pulmonary thromboembolism induced by intravenous infusion of collagen and epinephrine. Fig. 4B shows that all mice with the exception of one (14/15) died within 5 min of i.v. administration of collagen and epinephrine. In contrast, the two groups of aegyptin–treated mice showed a significant number of survivors (3 out of 15 at 0.5 mg/kg and 11 out 15 at 1.0 mg/kg of aegyptin).

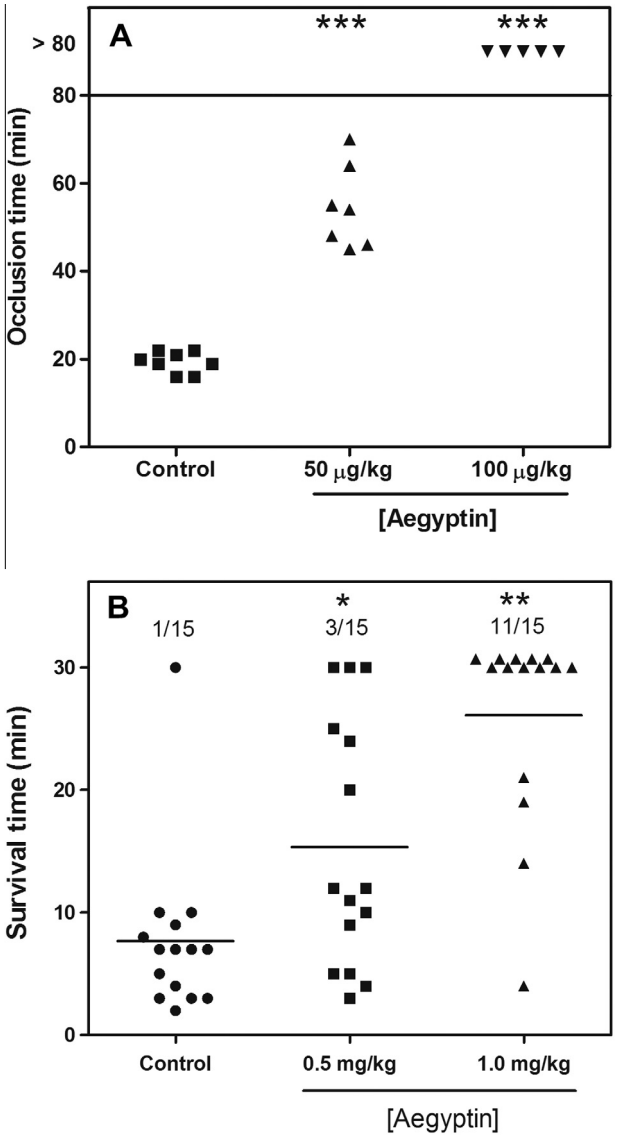

Fig. 4. Aegyptin prevents thrombus formation in collagen-dependent models. (A) Effect of aegyptin on a Rose Bengal/laser-induced model in rats. Either aegyptin (50 or 100 μg/kg) or PBS (control) was injected into the cava vein of rats, and thrombosis was induced by slow injection (over 2 min) of 90 mg/kg body weight of Rose bengal dye into the cava vein at a concentration of 60 mg/mL. Before injection, green light laser was applied to the site of injury from a distance of 3 cm and remained on for 80 min or until stable occlusion occurred. (B) Effect of aegyptin on a pulmonary thromboembolism model in mice. Mortality associated with an i.v. injection of collagen (0.8 mg/kg) and epinephrine (60 μg/kg) after administration of either PBS (control) or aegyptin. Animals that were alive 30 min after the challenge were considered to be survivors. Each symbol represents one individual. $^{*}P < 0.05$ vs control, $^{**}P < 0.01$ vs control.

4. Discussion

Aegyptin is a mosquito-derived protein that has been characterized as a high-affinity ligand to collagen, thus serving a potent inhibitor of platelet activation and aggregation [14]. Aegyptin interacts with high affinity with a peptide sequence based on the vWF-binding domain in collagen. It also interacts with low affinity (micromolar range) with peptides corresponding to the glycoprotein VI- and integrin alpha2beta1-binding sites in collagen [15]. As a result, aegytin can block platelet adhesion at high shear rates *in vitro*.

The recent observation that collagen fibers accelerate plasma clotting and enhance thrombin generation [3] led to evaluation of whether aegyptin could block this ability, which could contribute to its antithrombotic effect *in vivo*. Our data clearly demonstrate that aegyptin counteracts the collagen-induced acceleration of plasma clotting. This effect has been observed in experimental conditions employing either platelet-rich plasma or platelet-poor plasma supplemented with procoagulant phospholipids. It is concluded that the effect of aegyptin on plasma coagulation is completely independent of its effect on platelets.

A number of studies employing *in vivo* thrombosis models in mice have demonstrated a critical role for coagulation factor XII in thrombus formation [20–21]. Remarkably, factor XII deficiency confers protection against collagen-based models of induced thrombosis. Deficiency of either factor XII or factor XI also abrogates collagen-induced acceleration of plasma clotting *in vitro* [3]. Taken together it is proposed that collagen initiates the intrinsic pathway of coagulation *in vivo*, thus contributing to thrombin generation and fibrin deposition during thrombus formation. In

accordance with these studies, aegyptin was effective in inhibiting *in vivo* thrombus formation in two distinct models: 1) photochemically induced carotid artery thrombosis in rats and 2) pulmonary thromboembolism due to i.v. administration of collagen and epinephrine in mice. However, aegyptin was ineffective in preventing thrombus formation in an arteriovenous shunt model, which is not based on collagen exposure. We hypothesized that aegyptin inhibits thrombosis not only by blocking collagen-induced platelet responses but also by preventing the initiation of the intrinsic pathway of blood coagulation induced by collagen.

Thrombosis, the most common cause of death in the developed world, is a frequent result of atherosclerosis [22]. The rupture of atherosclerotic plaques promotes the exposure of subendothelial collagen fibers, which play a significant role in platelet deposition [23]. Kuijpers and colleagues have developed a new animal model that strongly suggests that collagen exposure in ruptured atherosclerotic plaques is essential for fibrin deposition [24]. Therefore agents that could simultaneously target the pro-aggregatory and procoagulant functions of collagen could be potential treatments for atherosclerosis-associated thrombosis.

In conclusion, we demonstrate that aegyptin is the first collagen binding protein that counteracts its ability to accelerate plasma coagulation. Additionally, aegyptin is an effective antithrombotic agent in models based on the collagen-induced hemostatic function. Taken together, our data indicate that aegyptin is a suitable molecule for *in vivo* studies aiming to elucidate the hemostatic role of collagen in both physiological and pathological conditions.

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