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Original Article

The effect of the fibrinolytic enzyme FII$_a$ from *Agkistrodon acutus* venom on acute pulmonary thromboembolism

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Aim: To evaluate the effects of the fibrinolytic enzyme FII$_a$ from *Agkistrodon acutus* venom on acute pulmonary thromboembolism (APT) in animal models.
Methods: Both rabbit and dog APT models were used. For the rabbit APT model, the thrombi weight before and after administration was measured. Central venous pressure (CVP) and mean arterial pressure (MAP) were measured before and 15, 30, 60, and 120 min after the injection of the blood clot. Partial thromboplastin time (APTT), prothrombin time (PT), platelet count, and fibrinogen concentration were measured using auto analyzers. Plasminogen activity was measured based on chromogenic substrates. In the dog APT model, pulmonary blood flow was recorded using pulmonary angiography.
Results: Intravenous administration of FIIa (0.1−5.0 mg/kg) improved the APT-induced hemodynamic derangements and reduced thrombi weight. The angiography evidence also showed that the pulmonary emboli had almost disappeared after FII$_a$ infusion. FII$_a$ (0.1, 0.5, or 1.0 mg/kg) did not impair the coagulation pathways, although very high doses of FII$_a$ (5.0 mg/kg) could stimulate the production of plasminogen and result in impairment of the pathways.
Conclusion: FII$_a$ could effectively protect against APT via degradation of thrombi with less activation of plasminogen, and may provide a novel fibrinolytic enzyme for targeting the main pathological processes of the disease.

Keywords: snake venom; fibrinolytic enzyme; pulmonary thromboembolism; angiography; plasminogen; central venous pressure; mean arterial pressure; urokinase; plasminogen

Introduction

Acute pulmonary thromboembolism (APT) is defined as an embolic occlusion of a pulmonary artery, which results in a sudden increase in pulmonary resistance, right ventricular afterload, and O$_2$ consumption with a reduction in right coronary artery perfusion[1]. Despite advances in diagnosis and therapy, the mortality associated with APT remains high[2, 3]. Various approaches are available for the treatment of APT, including anticoagulant drugs and thrombolytic agents. Anticoagulant therapy, such as heparin, is thought to enhance the effect on thrombolysis by preventing formation of new thrombi[4, 5]. However, anticoagulants have minimal effects on previously existing thrombi. Enhanced fibrinolysis prevents disturbances in the circulation to organs by dissolv-ing thrombi. It may be reasonable to assume that fibrinolytic therapy is effective against APT[6–10]. Current thrombolytic agents, such as tissue plasminogen activator (t-PA) and uroki-nase, which act as plasminogen activators, are effective at dissolving intravascular thrombi. These agents act indirectly on the thrombi by converting plasminogen, both circulating and fibrin-bound, into plasmin, which is the primary enzyme responsible for the removal of emboli. These conditions may "overflow" the systemic circulation and lead to systemic fibrinolysis and degradation of other clotting proteins[11], which may increase the bleeding tendency or may result in local activation of coagulation.

FII$_a$ is a novel fibrinolytic enzyme that is purified from Anhui *Agkistrodon acutus* venom and is a type of snake venom metalloproteinase. Based on its crystal structure, binding to ZnP^{2+} is known to be essential for it hydrolytic activity[12, 13]. We have demonstrated that FII$_a$ has the ability to directly degrade fibrin *in vitro* and effectively dissolve thrombi *in vivo*

without activating plasminogen or influencing the activities of t-PA and plasminogen activator inhibitor-1 (PAI-1)[14, 15]. These findings show that FII_a has a different mechanism of action from t-PA and urokinase. Additionally, upon examination of the tissue sections from kidney, liver, heart, and lung[16], the thrombolytic activities of FII_a did not lead to hemorrhage. Although many of the fibrinolytic properties of FII_a are known, there are few studies investigating its fibrinolytic effects on APT. In the present study, we mimicked clinical APT models and found that FII_a could ameliorate the hemodynamic derangements by degrading fibrin clots.

Materials and methods
Preparation of the enzyme FII_a
FII_a, the fibrinolytic enzyme that was isolated from *Agkistrodon acutus* venom, was prepared according to the method previously described by Liang *et al*[14].

Reagents
Urokinase was purchased from Sigma (St Louis, USA). The reagent pack for the plasminogen activity assay was obtained from Sun Biotechnology Company (Shanghai, China). All other reagents were of analytical grade and obtained from commercial sources.

Animals
All animal experiments were conducted in accordance with the National Guide for the Care and Use of Laboratory Animals and were approved by Sun Yat-sen University Animal Care and Use Committee (Guangzhou, China). Adult male New Zealand white rabbits (weight 2–3 kg, grade II) were supplied by the Experimental Animal Center of Zhongshan Medical College, Sun Yat-san University (China). Adult male Beagle dogs (weight 15–20 kg, grade II) were supplied by the Experimental Animal Center of Guangdong Province, China.

Experimental model and hemodynamic measurements in rabbits
The APT experimental model in the rabbit was performed according to the method described by Todd[17]. An autologous thrombus was produced in a 10-mL tube (ID=0.2 cm) by incubation at 37 °C for 2 h with constant agitation using a clinical rotor (160 r/min). Thrombus fragments (2 cm) were cut and weighed according to an established protocol. The extracted blood clots were injected into the lungs through the right ventricular catheter, which then embolized the pulmonary arteries. Mean artery pressure (MAP) and central venous pressure (CVP) were measured before and 15, 30, 60, and 120 min after the injection of the blood clot.

Treatment was started simultaneously with the injection of the blood clots through the left marginal ear vein. Seven different groups were established, each containing 10 animals as follows: treatment groups were injected with 0.1 mg/kg, 0.5 mg/kg, 1.0 mg/kg or 5.0 mg/kg FII_a in 20 mL of saline solution over a period of 2 h (at a rate of 10 mL/h); the embocontrol group was infused with saline solution (at a rate of 10 mL/h) instead of FII_a; the urokinase-control group was infused with 100 000 IU/kg over a period of 2 h (10 mL/h); and the remaining rabbits, which were neither injected with blood clots nor FII_a, were regarded as the sham-operated group and were infused with saline solution through the left marginal ear vein.

Two hours after the start of the experiments, the animals were sacrificed with an intravenous injection of 60 mg/kg pentobarbital sodium. The blood clots were carefully removed from the lungs and weighed.

Pulmonary angiography in dogs
This animal model was performed according to the methods described in Dias-Junior *et al*[18]. Under sterile dissection, a polyethylene catheter was placed into the right pulmonary artery through the right femoral vein. An autologous thrombus was produced in a 20-mL tube (ID=0.2 cm) by incubation at 37 °C for 2 h with constant agitation using a clinical rotor (160 r/min). Then, APT was induced by infusing the prepared clot into the lung through the polythene catheter. A pigtail catheter was repositioned in the pulmonary trunk. The extent of pulmonary embolism and the reperfusion rate were documented by the method of intra-arterial digital subtraction angiography (DFP-2000A, Toshiba) at 0, 30, and 60 min following the infusion of FII_a.

FII_a infusions were performed 2 h after the injection of the blood clots. Two different groups were established, each containing 6 animals as follows: (1) the dogs in the FII_a group were embolized animals that received FII_a infusions (1 mg/kg) over 1 h (at a rate of 10 mL/h); and (2) the dogs in the embocontrol group were embolized animals that were infused with saline solution instead of FII_a.

Sample collection and handling
Blood samples from rabbits were obtained immediately before and 2 h after blood clot injection through a catheter that was inserted into a femoral artery. Blood samples were collected in 3.8% sodium citrate (1:10 *v/v* citrate/blood) and centrifuged at 2000×*g* for 15 min at 4 °C. All samples were then stored at -70 °C until assayed.

Laboratory methods
An auto analyzer (Sysmex CA1500, Japan) was used to determine the partial thromboplastin time (APTT), prothrombin time (PT), and fibrinogen concentration. An automatic blood cell analyzer (Sysmex SE-9500, Japan) was used to determine platelet counts. Plasminogen activity was measured according to the reagent pack instructions based on chromogenic substrates.

Data analysis
Data at 2 h were converted to percentages, with a value of 100% corresponding to basal data, and were expressed as mean±SEM. One-way ANOVA, followed by Tukey's B test was used for multiple comparisons. A non-parametric test (Kruskal–Wallis H test) was used to determine the changes in the hemodynamic and biochemical parameters as well as to

compare the embo-control, FII$_a$ and urokinase-control groups. Differences with *P* values of less than 0.05 were considered to be statistically significant.

Results

Effect of FII$_a$ on coagulation in APT in rabbits

To elucidate the effect of FII$_a$ on APT, we systematically investigated its coagulation effects using a rabbit model. Table 1 summarizes the APTT, PT, platelet counts, fibrinogen concentration and plasminogen activity in normal rabbits, embo-control rabbits, FII$_a$ -treated rabbits and urokinase-treated rabbits. APTT, PT, and plasminogen values in the urokinase-control group were all significantly higher than those in the sham-operated group (*P*<0.05). However, the values for plasma levels of both fibrinogen concentration and platelet count were significantly lower than those of the normal rabbits (*P*<0.05). No changes were detected in the hemodynamic parameters that were measured 2 h post-FII$_a$ infusion (0.1, 0.5, or 1.0 mg/kg) compared with the sham-operated group. Although a significant decrease in fibrinogen and platelet count was observed, APTT, PT, and plasminogen activity increased in the animals that were treated with the highest dose of FII$_a$ (*P*<0.05, compared with sham-operated group). No blood loss was observed at the site of infusion in any treatment group.

Effect of FII$_a$ on APT-induced hemodynamic derangements in rabbits

Baseline hemodynamic parameters were similar in all experimental groups and showed no significant changes in the sham-operated animals throughout the study period (Figure 1). APT caused a sustained reduction in MAP and an increase in CVP in embolized animals, compared with the sham-operated group (*P*<0.05, Figure 1).

The animals treated with urokinase showed a decrease in CVP throughout the study period. An increase in MAP was observed 30 min following infusion of urokinase (*P*<0.05, compared with sham-operated group).

Two-hour measurements taken at all doses of infused FII$_a$ showed the following: a decrease in CVP throughout the study period (*P*<0.05, compared with the embo-control group, Figure 1); an increase in MAP for 60 min following the infusion of FII$_a$ in the 0.1 mg/kg and 0.5 mg/kg dose groups; and
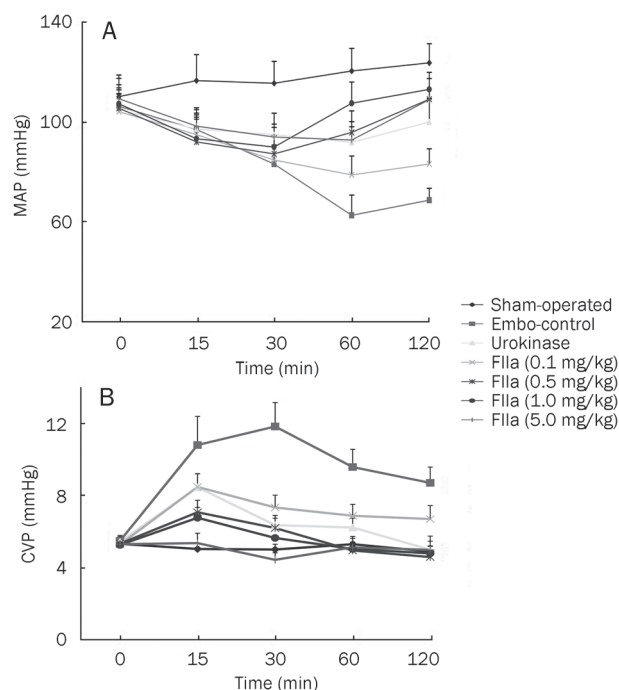

Figure 1. Effects of embolization and thrombolytic agents on mean arterial pressure (MAP, A) and central venous pressure (CVP, B). The data demonstrate that (1) embolization leads to an increase in CVP and a decrease in MAP and (2) FII$_a$ and urokinase consistently ameliorate the hemodynamic derangements.

an increase in MAP for 30 min following the infusion of FII$_a$ in the 1.0 mg/kg and 5.0 mg/kg dose groups (*P*<0.05, compared with the embo-control group, Figure 1).

Thrombolysis effect of FII$_a$ *in vivo*

The *in vivo* thrombolysis effect of FII$_a$ was studied in the rabbit APT model. No change in the thrombi weight was observed in the emboli-control group. The blood clots showed a weight loss of 79.38%±23.72% at 2 h post-infusion time point in the urokinase-treated group (Figure 2). FII$_a$ dose-dependently reduced thrombi weight, which ranged from 53.37%±18.67% for a 0.1 mg/kg dose to 91.36%±7.83% for a 1.0 mg/kg dose (Figure 2). No blood loss was observed in any of the animals treated with FII$_a$.

Table 1. Thrombolytic characteristics after infusion of urokinase and FII$_a$ in a rabbit APT model. Data were expressed as percentage of the basal value. *n*=10. Mean±SEM. [b]*P*<0.05 compared with the sham-operated group. APTT: partial thromboplastin time. PT: prothrombin time.

|	Fibrinogen (%)	Plasminogen (%)	APTT (%)	PT (%)	Platelet counts (%)
Sham-operated	101.28±14.27	97.68±20.74	101.36±6.85	99.29±17.84	101.40±10.19
Embo-control	105.37±13.39	103.83±18.65	99.54±16.16	98.48±12.22	100.83±12.61
Urokinase	69.14±26.09[b]	180.58±30.96[b]	283.12±40.37[b]	242.87±37.63[b]	59.82±19.07[b]
FII$_a$(0.1 mg/kg)	107.51±20.07	104.48±24.57	97.38±10.31	101.69±11.06	99.52±8.52
FII$_a$(0.5 mg/kg)	93.41±15.56	109.62±26.74	100.59±9.63	104.19±6.72	93.84±11.93
FII$_a$(1.0 mg/kg)	89.37±18.92	115.35±24.90	104.62±11.49	103.94±14.27	90.81±13.29
FII$_a$(5.0 mg/kg)	75.31±24.15[b]	143.37±31.42[b]	173.80±30.62[b]	143.64±25.33[b]	70.35±18.98[b]
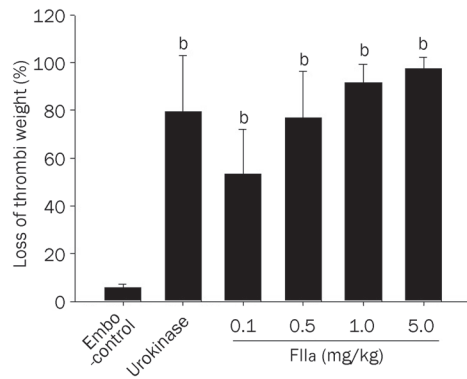
Figure 2. Effect of FII$_a$ on thrombi weight in rabbits (*n*=10 in each group). Dose-dependent reduction in thrombus weight by FII$_a$ infusion. Data were expressed as mean±SEM. ^{b}*P*<0.05, compared with embo-control.

Pulmonary angiography in the Beagle APT models

Most pulmonary emboli occurred in the lower lobes of the right lung. Serious obstructions occurred in the branches of the right lower lobar pulmonary artery in the embo-control group, following a significant decrease in the perfusion of the right lower lobes of the lung (the reperfusion rate was 0.6%±0.07%, Figure 3). Administration of 1 mg/kg of FII$_a$ resulted in the gradual disappearance of the emboli. Thirty minutes after the infusion of FII$_a$, a lower level of obstruction was detected in the branches of the lower lobar pulmonary

Figure 3. Digital subtraction angiography in APT. Arrows indicate thrombi in the right pulmonary artery. (A) Before pulmonary thromboembolism; (B) Two hours after pulmonary thromboembolism. The right pulmonary artery shows a complete obstruction. (C) FII$_a$ treatment for 30 min. A reduction in obstruction was detected in the lower lobar pulmonary artery branches. (D) FII$_a$ treatment for 60 min. The pulmonary emboli almost completely disappeared.

artery, and the reperfusion rate was 70.41%±7.62% (Figure 3). Sixty minutes after the infusion of FII$_a$, the pulmonary emboli had almost completely disappeared, and the reperfusion rate of the upper to lower lobe was 85.34%±8.42% (Figure 3).

Discussion

In the present study, we reported that FII$_a$ from *Agkistrodon acutus* venom demonstrated significantly protective properties in APT induced by the injection of preformed blood clots. An improvement in the APT-induced hemodynamic derangements and a reduction in thrombi weight were observed. The angiography evidence in the dog APT model also showed that the pulmonary emboli almost completely disappeared with the infusion of FII$_a$. In addition, FII$_a$ did not impair the coagulation pathways.

APT can produce severe cardiopulmonary dysfunction that is characterized by pulmonary arterial hypertension, right ventricular failure, and hypoxemia, which is the principal cause of APT-related death[19-22]. In addition, the major adverse effect is attributed to the direct mechanical obstruction of the pulmonary arteries. This evidence suggests that promoting fibrinolysis might be a promising target for therapeutic strategies for APT[23].

In this study, we found that lung embolization resulted in typical changes associated with APT that were characterized by a significant decrease in MAP and a dramatic increase in CVP, which were consistent with the results previously described by Todd *et al*[17]. The angiography evidence in the dog APT model also showed that the pulmonary blood flow was almost completely occluded 2 h after pulmonary embolization.

Using the rabbit model of APT, we found that all doses of FII$_a$ not only significantly attenuated the increase in CVP and the decrease in MAP but also effectively reduced the thrombi weight in the pulmonary artery. Furthermore, the angiography evidence in the dog APT model also showed that the pulmonary emboli almost completely disappeared after a 60-min FII$_a$ infusion, and pulmonary blood flow was almost fully returned to baseline levels. In our previous study[14, 15], we found that fibrin clots were hydrolyzed more efficiently by FII$_a$ than by urokinase *in vitro*. Because FII$_a$ is a snake venom fibrinolytic enzyme, its proteolytic action should involve the mechanism of directly degrading fibrin. Furthermore, FII$_a$ lysed fibrin by direct proteolysis without activating intrinsic plasminogen[24, 25], which resulted in a greater effectiveness than urokinase. These findings suggest that FII$_a$ could be beneficial in improving APT-induced hemodynamic derangements by its direct fibrinolytic effect on thrombi.

Because of the coagulation abnormalities, bleeding is a major complication of thrombolytic therapy. The risk of hemorrhage usually occurs at a site of previous surgery or puncture[26, 27]. As a measure of ongoing coagulation during the infusion of the agents, we determined the fibrinogen concentration, platelet counts, plasminogen activity, PT and APTT.

In this study, the significant changes in these coagulation parameters in the urokinase-treated group indicated that infu-
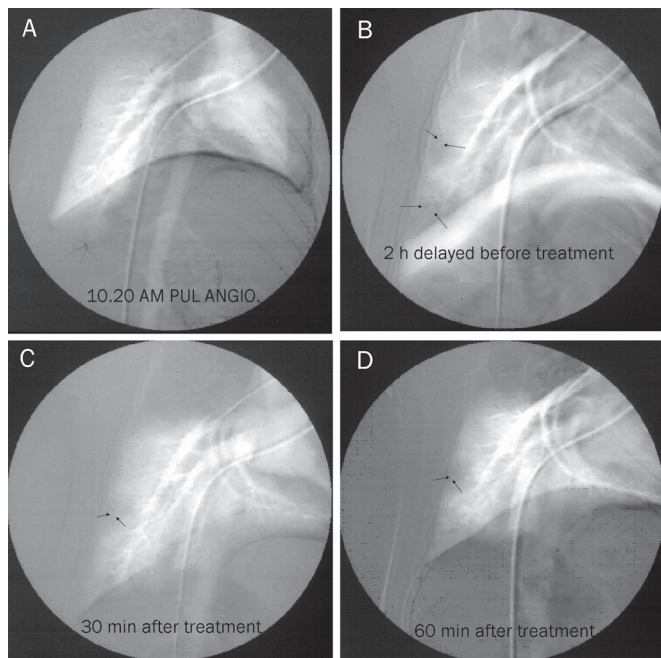
sion of urokinase impaired the intravascular coagulation in the animals. After a 2-h FII$_a$ infusion (0.1, 0.5, or 1.0 mg/kg), no significant changes in the parameters of coagulation were detected, and no blood loss was observed at the site of infusion. These results suggest that intravascular administration of FII$_a$ did not impair the coagulation system and produced no hemorrhages. FII$_a$ effectively decreased the thrombi weight in a rabbit APT model. Because FII$_a$ does not activate plasminogen, many complex secondary effects, such as platelet activation and fibrinogen degradation, are avoided. The advantages of the plasminogen-independent fibrinolysis induced by FII$_a$ was further demonstrated by the findings of no bleeding in any of the rabbits treated with FII$_a$ in our previous studies[13, 14] and a much wider therapeutic window compared with urokinase.

However, it should be emphasized that FII$_a$, at its extremely high dose, may result in internal hemorrhaging in some animals. In this study, the values of plasminogen activity, fibrinogen concentration, platelet count, APTT, and PT showed significant changes compared to the respective baseline values in the group with the highest dose of FII$_a$. Our previous study showed that FII$_a$ influenced blood coagulation by inhibiting the platelet aggregation that was induced by adenosine phosphate and degradation of prothrombin and factor X[13]. Plasminogen provides an important control of fibrinolysis by its degradation of blood coagulation proteins[28]. In addition to its fibrinolytic activity, FII$_a$ is also involved in the degradation of gelatin and collagen, which are the main proteins that compose the vascular basement membrane. Plasminogen also activates collagenases and disrupts the basement membrane barriers[29]. This evidence suggests that extremely high dose of FII$_a$ might stimulate the production of plasminogen *in vivo*, which may be the reason for the impairment of the coagulation pathways. These results suggest that if FII$_a$ is employed in the treatment of APT, it should be utilized in a manner that does not cause systemic activation of the fibrinolytic system. Additional work is needed to further characterize this issue.

In conclusion, FII$_a$ may have protective effects on TAPT by it direct degradation of fibrin clots. These therapeutic effects support the hypothesis that FII$_a$ could be used for reducing thrombi, restoring blood flow in the lung, and improving cardiovascular function. However, we emphasize that the true utility of FII$_a$ in combating APT will require further direct testing in clinical trials.

Acknowledgements

Author contribution

Jia-shu CHEN and Guang-mei YAN designed the study; Xi LIN, Xiu-xia LIANG and Peng-xin QIU performed the research; Jian-jun TANG contributed new analytical reagents and tools; and Xi LIN analyzed the data and wrote the paper.

Abbreviations

APT, acute pulmonary thromboembolism; MAP, mean arterial pressure; CVP, central venous pressure; APTT, partial thromboplastin time; PT, prothrombin time; PAI-1, plasminogen activator inhibitor-1.

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