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A novel fibrinolytic enzyme, FII_a, was isolated from *Agkistrodon acutus* venom, which can degrade fibrin/fibrinogen and dissolve thrombus without activating plasminogen or influencing the activities of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type-1 (PAI-1). In this study, we evaluated the effect of FII_a on lipopolysaccharide (LPS)-induced experimental disseminated intravascular coagulation (DIC) in rabbits, through the continuous infusion of 100-μg/kg/h LPS for a period of 6 h. Seven groups were established: LPS control, FII_a (0.1, 0.3, and 0.6 mg/kg/h, respectively), heparin control (100 IU/kg/h), heparin + FII_a (heparin 100 IU/kg/h associated with FII_a 0.3 mg/kg/h), and a saline control group. A continuous injection of LPS induced a gradual impairment in hemostatic parameters, kidney fibrin deposition, and a high mortality rate. The intravenous administration of FII_a improved the concentration of fibrinogen, the activities of protein C, plasminogen, t-PA, antithrombin III (ATIII), and PAI-1. Kidney fibrin deposition and the mortality also decreased. In the *in vitro* experiments, FII_a can degrade fibrin/fibrinogen and high-dose FII_a enhanced the activity of protein C. These findings suggest that the effects of FII_a on LPS-induced DIC were from fibrinogen degradation and enhanced protein C activity. The simultaneous administration of FII_a and heparin further improved all the hemostatic parameters, including decreased kidney fibrin deposition, and none of the rabbits died within 24 h, which indicates that the effects were mediated by degradation of fibrin/fibrinogen together with thrombin inhibition. We conclude that FII_a may be useful in the treatment of DIC. (Translational Research 2007;150:295–302)

Abbreviations: ATIII = antithrombin III; DIC = disseminated intravascular coagulation; LPS = lipopolysaccharide; PAI-1 = plasminogen activator inhibitor type-1; t-PA = tissue plasminogen activator

Disseminated intravascular coagulation (DIC) is secondary to an underlying disorder, which can be defined as an acquired syndrome characterized by the activation of intravascular coagulation and subsequent intravascular fibrin formation. This process may be accompanied by secondary fibrinolysis, which often leads to a bleeding tendency, or deficient fibrinolysis,[1,2] which frequently results in organ failure caused by the disturbance of microcirculation by numerous microthrombi formed in many organs.[3,4] This process is often a serious complication and a cause for the poor prognosis in DIC. Although the bleeding tendency can be treated with replacement therapy, such as platelet concentrates and plasma, the intravascular fi-

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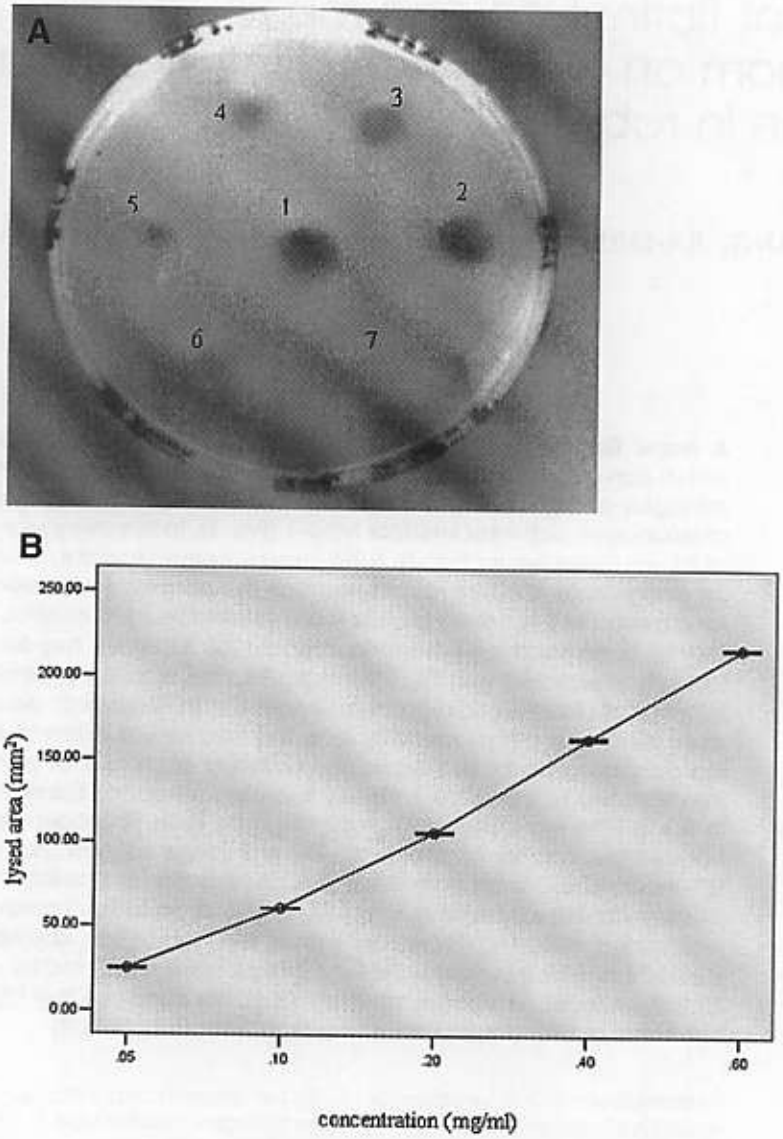

Fig 1. Fibrinolytic activity of FII_a. **A**, The lysed area of different concentrations. 1–5: FII_a after purified 0.6, 0.4, 0.2, 0.1, 0.05 mg/mL, respectively; 6: Urokinase 5000 IU/mL; 7: NS. **B**, The lysed area was obtained by using the human fibrin plate method. The activities were expressed as the diameter product (mm^2) (mean ± standard deviation, n = 3).

brin formation and organ failure are often irreversible. Anticoagulant therapy such as heparin is thought to enhance the effects of thrombolysis by preventing formation of new fibrin. However, anticoagulants cannot always prevent the progressive organ failure as enhanced fibrinolysis is a defense mechanism to prevent the disturbance of microcirculation in organs by dissolving microthrombi. Thus, it may be reasonable to assume that fibrinolytic therapy is effective for DIC.[5,6] Current thrombolytic agents such as tissue plasminogen activator (t-PA) and urokinase act as plasminogen activators that are effective at dissolving intravascular

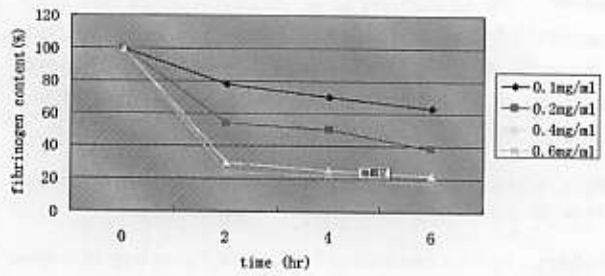

Fig 2. Fibrinogenolytic activity of FII_a. Fibrinogen was determined in human plasma after incubation with FII_a at 2, 4, and 6 h. The results were expressed as % (n = 3).

Table I. Hemostatic parameters 2 and 6 h after LPS infusion into rabbits in different treatment groups

Group	Time (h)	Fibrinogen (%)	Protein C (%)	ATIII (%)	Plasminogen (%)	t-PA (%)	PAI-1 (%)
Saline control	2	102.65 ± 8.36†	101.11 ± 8.30†	99.95 ± 4.89*	104.12 ± 19.12†	102.53 ± 23.34*	109.04 ± 8.02†
	6	95.90 ± 10.87†	101.57 ± 10.61†	103.28 ± 8.76†	136.08 ± 30.92†	101.32 ± 8.03†	104.76 ± 9.48†
LPS control	2	81.13 ± 17.84	61.83 ± 24.14	86.57 ± 14.57	60.59 ± 32.00	77.62 ± 18.06	161.48 ± 23.10
	6	48.95 ± 21.47	52.22 ± 20.66	57.51 ± 15.73	37.93 ± 19.42	51.08 ± 26.97	172.29 ± 29.75
Heparin control	2	82.14 ± 15.77	76.85 ± 20.17	84.75 ± 10.37	70.53 ± 20.45	80.03 ± 17.87	160.37 ± 20.27
	6	70.45 ± 16.33*	75.73 ± 15.90*	68.36 ± 12.37	60.84 ± 18.34*	72.45 ± 24.90	140.21 ± 22.81*
FII_a (0.1 mg/kg/h)	2	94.18 ± 16.32	92.97 ± 26.44*	98.55 ± 7.73*	109.84 ± 34.24†	103.20 ± 31.83*	119.35 ± 18.48†
	6	73.87 ± 13.58†	90.96 ± 23.98†	96.36 ± 12.21†	92.84 ± 31.46†	114.31 ± 30.73†	94.55 ± 30.36†
FII_a (0.3 mg/kg/h)	2	84.71 ± 13.29	109.06 ± 34.30†	99.13 ± 5.62*	125.14 ± 39.67†	82.33 ± 35.74	93.32 ± 34.62†
	6	80.04 ± 15.99†	112.32 ± 34.73†	98.94 ± 10.94†	128.17 ± 33.35†	87.23 ± 32.02*	94.55 ± 30.36†
FII_a (0.6 mg/kg/h)	2	88.41 ± 31.04	100.06 ± 31.48*	100.30 ± 10.22*	127.85 ± 39.86†	108.40 ± 19.76†	125.34 ± 15.08†
	6	38.24 ± 13.41	126.04 ± 29.49†	103.80 ± 4.14†	177.50 ± 32.64†	110.05 ± 33.74†	107.74 ± 10.73†
FII_a + heparin	2	80.77 ± 12.43	105.04 ± 22.33†	97.93 ± 4.69	120.37 ± 30.78†	83.77 ± 26.68	98.40 ± 25.66†
	6	79.37 ± 14.45†	110.49 ± 24.70†	109.14 ± 8.93†	124.18 ± 32.43†	98.20 ± 27.09†	97.43 ± 29.04†

Note: Data shown as mean ± SD percent of the basal value, n = 10.
*$P < 0.05$ compared with the LPS control group.
†$P < 0.01$ compared with the LPS control group.

microthrombi. These agents act on the microthrombi indirectly by activating plasminogen, circulating plasminogen as well as fibrin-bound plasminogen, which is the primary enzyme responsible for removal of fibrin deposits. It has also been observed that circulating concentrations of plasminogen activator inhibitor type-1 (PAI-1) increase during DIC. Increased PAI-1 has been associated with a predisposition to thrombosis, which is a specific inhibitor of t-PA released from endothelial cells. These conditions may "overflow" the systemic circulation that leads to systemic fibrinolysis and degradation of other clotting proteins,[7] increases the bleeding tendency, or results in local activation of coagulation caused by the insufficient administration of thrombolytic agents.

FII_a is a novel fibrinolytic enzyme purified from Anhui *Agkistrodon acutus* venom, which is a member of snake venom metalloproteinase. Based on its crystal structure, binding to the Zn^{2+} was found to be essential for hydrolytic activity.[8,9] *In vitro* FII_a can directly degrade α-chains and β-chains of fibrin/fibrinogen, whereas *in vivo* studies show it dissolves thrombus without activating plasminogen or influencing the activities of t-PA and PAI-1.[10] This shows that FII_a has a different mechanism of action from t-PA and urokinase. Additionally, in the examination of tissue sections from kidney, liver, heart, and lung,[11] the thrombolytic activities of FII_a lacked hemorrhaging. These results suggest that FII_a may be a safe and attractive agent for treating DIC.

In this study, we determined the effect of FII_a on a lipopolysaccharide (LPS)-induced model of DIC and investigated the mechanism of its action in an attempt to gain a better understanding of its clinical potential of FII_a.

MATERIALS

Snake venoms. *Agkistrodon acutus* venom lyophilized was collected from Qimen Snake Farm (Anhui, China) and stored in desiccators.

Reagents LPS, heparin, and human fibrinogen (95% clottable) were purchased from Sigma (St Louis, Mo). The fibrinogen concentration determination reagent pack (Clauss method) and the reagent packs for the activity assays of antithrombin III (ATIII), protein C, plasminogen, PAI-1, and t-PA were obtained from Sun Biotechnology Company (Shanghai, China); all other reagents were analytical grade from commercial sources.

Animals. Adult male New Zealand white rabbits (weight 2–3 kg, Grade II, Certificate 2001A033) were supplied by the Experimental Animal Center of Zhongshan Medical College, Sun Yat-san University.

METHODS

Purification of the enzyme. FII_a, the fibrinolytic enzyme isolated from *Agkistrodon acutus* venom, was prepared according to the method previously described.[9]

Fibrinogenolytic and fibrinolytic activities. Fibrinogenolytic activity was measured by fibrinogen content in human plasma at various time intervals, namely 2, 4, and 6 h; when incubated with a different amount of FII_a (FII_a 10 μL at several different concentrations, 0.1, 0.2, 0.4, 0.6 mg/mL), the determination of fibrinogen content used the method of Rampling and Gaffeny.[12]

Fibrinolytic activity used a fibrin plate technique as described by the method of Astrup and Müllertz.[13] FII_a 10 μL at several different concentrations, 0.05, 0.1, 0.2, 0.4, and 0.6 mg/mL, were applied. Urokinase given 5000 IU/mL was used as positive control; each concentration was tested 3 times. NS was used as negative control.

Experimental models. All procedures were conducted according to the ethical guidelines of the Animal Care and Use

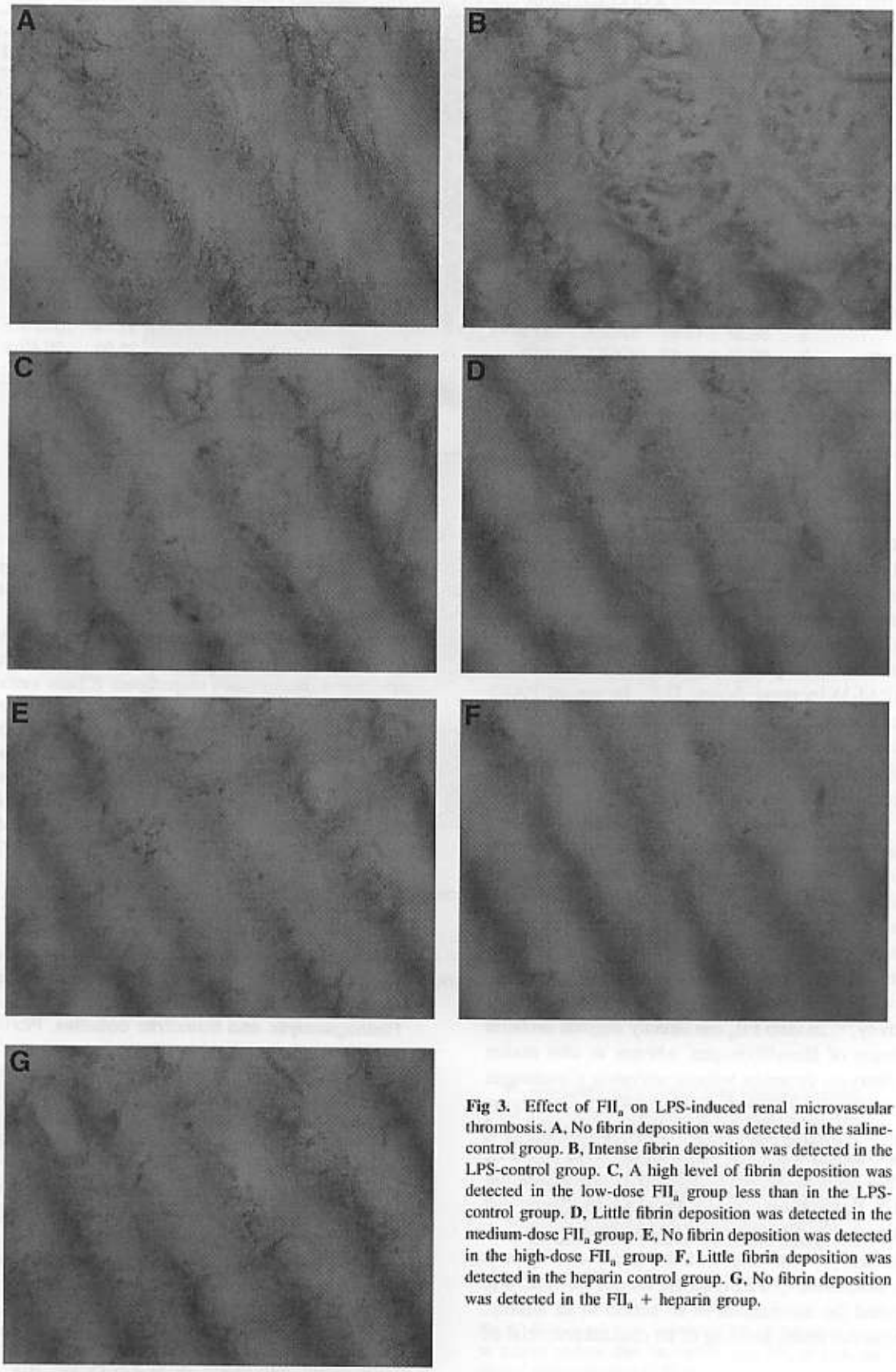

Fig 3. Effect of FII_a on LPS-induced renal microvascular thrombosis. **A**, No fibrin deposition was detected in the saline-control group. **B**, Intense fibrin deposition was detected in the LPS-control group. **C**, A high level of fibrin deposition was detected in the low-dose FII_a group less than in the LPS-control group. **D**, Little fibrin deposition was detected in the medium-dose FII_a group. **E**, No fibrin deposition was detected in the high-dose FII_a group. **F**, Little fibrin deposition was detected in the heparin control group. **G**, No fibrin deposition was detected in the FII_a + heparin group.

Committee at SUN Yat-Sen University. DIC experimental models were performed by the method of Jose Hermida,[14] which were induced by infusing LPS in 60 mL of saline solution at a rate of 100 μg/kg/h (10 mL/h) through the marginal ear vein of rabbits over a period of 6 h. Animals were anesthetized by an intramuscular injection of 30-mg/kg ketamine hydrochloride, followed by intramuscular supplements of ketamine hydrochloride given throughout the experiment.

Treatments started simultaneously with LPS infusion through the contralateral marginal ear vein. Seven different groups were established, containing 10 animals each: Treatment groups (low-, medium-, and high-dose FII_a) were given 0.1, 0.3, and 0.6 mg/kg/h in 60 mL of saline solution over a period of 6 h (10 mL/h). The LPS control group was infused with saline solution (at a rate of 10 mL/h) over a period of 6 h. The heparin control group was infused with heparin at a rate of 100 IU/kg/h (10 mL/h) over a period of 6 h. Heparin + FII_a group was infused with 100-IU/kg/h heparin + 0.3-mg/kg/h FII_a (at a rate of 10 mL/h) over a period of 6 h. The additional rabbits, which were given neither LPS nor FII_a, were infused with saline solution through both marginal ear veins.

Surviving rabbits were sacrificed at 24 h after the start of each experiment with an intravenous injection of 60-mg/kg pentobarbital sodium. The right kidneys were extracted from animals (survivors and nonsurvivors) for subsequent histologic study.

Sample collection and handling. Blood samples were taken through a catheter inserted into a femoral artery immediately before LPS infusion and at 2 and 6 h postinfusion. Blood samples were collected in 3.8% sodium citrate (1:10 vol/vol citrate/blood). The blood was centrifuged at 2000×*g* for 15 min at 4°C. Blood for the measurement of t-PA was collected in Stabilyte tubes (Biopool, Umea, Sweden) in order to avoid the interference with its inhibitors. All samples were stored at −70°C until assayed.

Laboratory methods. Fibrinogen concentration was measured according to the method of Clauss. ATIII, protein C, plasminogen, PAI-1, and t-PA activity were measured according to the reagent pack instruction based on chromogenic substrates.

Tissue preparation for histologic examination. Kidney tissue specimens were fixed in formalin, embedded in paraffin, stained with phosphotungstic acid-hematoxylin stain, and examined for the presence of fibrin microthrombi by a pathologist.

Data analysis. Data at 2 and 6 hours were converted to percentages, with a value of 100% assumed for basal data and was expressed as mean ± standard error of the mean. Student *t* test was used to seek differences between the LPS control and saline solution control groups. One-way analysis of variance, followed by the Tukey *B* test for multiple comparisons, was used to compare the LPS and FII_a heparin and heparin+ FII_a groups. Differences in mortality at 6 and 24 h were assessed with the Fisher exact test. Differences with *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

Fibrinogenolytic and fibrinolytic activities. Fibrinolytic activity of FII_a was determined on fibrin plate formed by human plasma. Increasing concentration of FII_a demonstrated a dose-dependent lysis (Fig 1). Fibrinogenolytic activity of FII_a of different concentrations was also determined (Fig 2). The amounts of fibrinogen remaining in plasma were determined at 2, 4, and 6 h.

LPS-induced DIC. Two-hour post-intravenous injection of LPS (100 μg/kg/h) into rabbits caused a significant decrease in the concentration of fibrinogen and the activities of protein C, ATIII, plasminogen, and t-PA ($P < 0.05$, compared with the saline solution group). However, PAI-1 activity increased under similar experimental conditions ($P < 0.05$). At 6 hours postinjection, fibrinogen concentration and the activities of protein C, plasminogen, t-PA, and ATIII decreased ($P < 0.05$), whereas PAI-1 activity increased ($P < 0.05$, Table I), which is consistent with the data obtained at 2 h. Intense fibrin deposition was detected in most LPS-treated rabbits (Fig 3). Five of the 10 rabbits in the LPS control group died within the first 6 h, and 9 died within 24 h.

Evaluations of drugs. ***Effects of FII_a on DIC.*** We found that infusion of a low, medium, and high dose of FII_a ameliorated the activities of protein C, ATIII, plasminogen, and PAI-1 at 2 h when compared with LPS control group ($P < 0.05$). Six-hour measurements made at all doses of infused FII_a showed a significant increase in the activities of protein C, plasminogen, t-PA, and ATIII ($P < 0.05$), whereas PAI-1 activity decreased ($P < 0.05$). Low and medium doses of FII_a also ameliorated fibrinogen concentration. The activities of protein C and plasminogen were increased at the high-dose FII_a group ($P < 0.05$, Table I), compared with the saline control group.

A high level of fibrin deposition was detected in most of the low-dose FII_a group, whereas a lower layer of fibrin deposition was detected in most of the medium-dose FII_a group. No fibrin deposition was detected in most of the high-dose FII_a group (Fig 3). Two of the 10 rabbits in the low-dose FII_a group died within the first 6 h, and 8 died within 24 h. However, none of the 10 rabbits in the medium- and high-dose groups died within the first 6 h, whereas 5 died within 24 h. The mortality rates in the medium- and high-dose groups were significantly lower than in the LPS control group ($P < 0.05$).

Effect of heparin on DIC. No changes were detected in hemostatic parameters measured 2 h post-heparin infusion, when compared with the LPS control group. Measurements made at 6 h postinfusion showed a significant increase in fibrinogen concentration and higher activities of protein C and plasminogen ($P < 0.05$), whereas PAI-1 activity decreased ($P < 0.05$, Table I). Little fibrin deposition was detected in most of the treated heparin group (Fig 3). Two of the 10 rabbits in

the heparin treated group died within the first 6 h, and 7 died within 24 h.

Effect of FII_a + heparin on DIC. A significant increase in the activities of protein C, plasminogen ($P < 0.05$) was observed at 2 h postinfusion with both FII_a and heparin, when compared with the LPS control group. A decrease in PAI-1 activity was found at 2 h ($P < 0.05$). Similar changes were also observed at the 6-h time point ($P < 0.05$, Table I). No fibrin deposition was detected in most of the FII_a + heparin group (Fig 3), and no mortality was observed in the FII_a + heparin group 24 h postinjection.

DISCUSSION

LPS, a constituent of the outer membrane of the gram-negative bacteria, is a major pathogenic factor contributing to the initiation of DIC in humans and animals. Induction of DIC leads to the generation of cytokines by monocytes and endothelial cells, which activate coagulation and fibrinolytic pathways.[15] In this study, the administration of LPS resulted in typical changes of DIC characterized by a significant decrease in the activities of plasminogen, ATIII, protein C, t-PA, and fibrinogen concentration. A dramatic increase in PAI-1 activity, intense kidney fibrin deposition, and a high mortality observed in this study are consistent with the results described previously by Jose Hermida et al.[14–16]

Using this rabbit model of DIC, we found that all 3 doses of FII_a administered could not only improve the activities of plasminogen, ATIII, protein C, t-PA, PAI-1, and the concentration of fibrinogen ($P < 0.05$), but also could decrease the mortality of animals treated with medium- and high-dose FII_a ($P < 0.05$). This dramatic benefit was further verified by a significant reduction in fibrin deposition observed in the histologic kidney section.

The initial decrease in protein C and/or ATIII may have particular prognostic significance in the clinical management of DIC, with almost absolute lethality observed among DIC.[17–22] In this study the improvements in protein C and ATIII activity by FII_a were remarkable among the changes observed in coagulation-related parameters. Furthermore with increasing dosage of FII_a, the activity of protein C increased over baseline. The chief cause of protein C and ATIII deficiency in LPS-induced DIC is not a decrease in production but enhanced generation of thrombin.[23] Our previous study showed that FII_a influenced blood coagulation by inhibiting platelet aggregation induced by adenosine phosphate and degradation of prothrombin and factor X.[24] Protein C provides important control of blood coagulation by regulating the activities of factor VIII and factor V, which are cofactors in the activation of factor X and prothrombin, respectively. In addition to anticoagulant activity, FII_a is also related to the degradation of gelatin and collagen,[24] whereas activated protein C can activate gelatinase A.[25] This evidence suggests that such a dose might have succeeded in enhancing the activity of protein C. Additional work is needed to assess the effect of FII_a on the coagulation changes occurring.

A severe reduction in anticoagulant capacity and inhibition of fibrinolysis are opposed to a massive activation of coagulation, which leads to overwhelming fibrin formation. Abundant intravascular fibrin formation results in microvascular thrombosis causing widespread organ ischemic. The kidneys are thought to be especially prone to fibrin formation in DIC. In the LPS-induced DIC model, FII_a did influence fibrinolysis concomitant with the changes in the activities of PAI-1, t-PA, and plasminogen. In addition, such treatments with FII_a reduced kidney microvasscular thrombosis induced by LPS. When compared with the respective baseline values, t-PA and PAI-1 activity remained unchanged, which suggests that FII_a has no direct effect on t-PA and PAI-1 activity.[9] Protein C has a secondary antithrombotic action by forming complexes with PAI-1 to prevent its inhibition of fibrinolysis,[24] and the activity of protein C was found to be enhanced by FII_a. Therefore, the improvement in the activities of t-PA and PAI-1 by FII_a could be partly explained by the enhanced activity of protein C. It was interesting that the activity values of plasminogen showed a significant change with the respective baseline values in the high-dose FII_a group. In a previous study FII_a had no direct effect on plasminogen *in vitro*.[9] It seems likely that FII_a might stimulate the production of plasminogen *in vivo*. Additional work is needed to assess this issue.

FII_a can improve the fibrinogen concentration caused by LPS-induced DIC and reduce kidney deposition. Although FII_a can degrade fibrinogen/fibrin *in vitro*,[9] previous *in vitro* experiments have demonstrated that FII_a degraded more fibrinogen than urokinase but *in vivo* experiments had disparate results where less fibrinogen was degraded compared with urokinase.[26] Collectively, these data propose a model that the improvement of fibrinogen and the reduction of fibrin deposition are caused by the degradation of fibrinogen/fibrin and the dissolution of the microvascular thrombosis by FII_a.

In this investigation, FII_a reduced the mortality caused by LPS-induced DIC. DIC can have widespread activation on blood coagulation, which results in the intravascular formation of fibrin. This process may lead to thrombotic occlusion of small and mid-sized vessels that contribute to multiple organ failure. These vessels have been considered the important causes of DIC

death. Furthermore, protein C also plays a role in pathogenesis of microthrombosis. In the light of our data, FII_a not only degrades fibrinogen and dissolves the microvascular thrombosis *in vivo*, but it may also enhance the activity of protein C. These findings suggest that FII_a can have a benefit in improving LPS-induced DIC by degrading fibrinogen and enhancing the activity of protein C.

We also analyzed the effects of FII_a and heparin on DIC. Heparin, the traditional anticoagulant used for modulation of the DIC process, had variable effects on both prophylaxis and treatment. When rabbits were treated with heparin alone, hemostatic parameters were improved. However, mortality was not significantly reduced, which suggests that lethality cannot be explained merely by hemostatic imbalance.

The simultaneous administration of medium-dose FII_a and heparin induced further improvement of the hemostatic parameters elicited by each drug alone. The most magnetic result was that none of the rabbits with simultaneous administration of FII_a and heparin died when compared with the animals given heparin alone (7/10 died) or FII_a alone (5/10 died in the medium- and high-dose FII_a groups). From these experiments, FII_a and heparin given simultaneously has a more effective result than medium-dose FII_a or heparin alone for DIC. Effective degradation of fibrinogen/fibrin together with the thrombin inhibition would be sufficient to explain these results.

In conclusion, FII_a can reduce LPS-induced DIC mortality not only by its effects on anticoagulant activity but also by its fibrinolytic activity. The simultaneous administration of FII_a and heparin can be a more efficient treatment toward LPS-induced DIC.

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