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Identification of a nitric oxide-dependent hypotensive effect of anticoagulation factor II from the venom of *Agkistrodon acutus*

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ABSTRACT

Anticoagulation factor II (ACF II) isolated from the venom of *Agkistrodon acutus* is a member of the coagulation factor IX/coagulation factor X-binding protein (IX/X-bp) family. ACF II forms a 1:1 complex with activated coagulation factor X in a Ca^{2+}-dependent fashion and thereby blocks the amplification of the coagulation cascade. In the present study, we have investigated the effect of ACF II on the mean arterial blood pressure (MABP) and heart rate (HR) in anaesthetized rats. The results indicate that ACF II induces a dose-dependent response in rats with a short fast drop of MABP followed by an increase and then a longer lasting slight decrease in MABP, but does not obviously affect HR. ACF II-induced hypotension is significantly blocked by the nitric oxide (NO) synthase inhibitor N-omega-L-arginine methyl ester (L-NAME). ACF II produces a concentration-dependent relaxation of rat aortic rings with functional-endothelium. The ACF II-induced vasodilatation is completely inhibited by removal of endothelium and significantly inhibited by pretreatment with L-NAME. These observations demonstrate that ACF II induces hypotension through an endothelium-dependent vasodilation, which is strongly mediated by the release of NO from endothelium. ACF II exhibits high anticoagulation activity in vivo based on activated partial thromboplastin time assay. Therefore, ACF II is so far identified as the first unique bifunctional protein in the IX/X-bp family that has both anticoagulant and hypotensive effects on the blood of rats through different pathways.

1. Introduction

A family of coagulation factor IX/factor X-binding proteins (IX/X-bps) has been found from snake venoms [1,2]. The proteins of this family have high homologous sequences, and form 1:1 complexes with coagulation factor IX (FIX)/activated coagulation factor IX (FIXa) or coagulation factor X (FX)/activated coagulation factor X (FXa) in a Ca^{2+}-dependent fashion and prolong the clotting time [3–5]. IX/X-bps bind to the γ-carboxyglutamic acid domain of FIX or FX in a Ca^{2+}-dependent manner, thus blocking the amplification of the coagulation cascade. Therefore, these proteins function as anticoagulants. The crystallographic structures of habu IX/X-bp [6], habu IX-bp [7] from habu snake venom and Deinagkistrodon X-bp [3] from *Deinagkistrodon* venom are very similar.

Anticoagulation factor I (ACF I) and anticoagulation factor II (ACF II) purified from the venom of *Agkistrodon acutus* are members of the IX/X-bp family, because they form a 1:1 complex with FXa in a Ca^{2+}-dependent fashion with marked anticoagulant activity [8,9]. ACF I and ACF II, as naturally occurring nonenzymatic anticoagulants, are devoid of proteolytic, esterolytic, L-amino acid oxidase, phospholipase A, thrombin-like, fibrinolytic and hemorrhagic activities. Both proteins are composed of two peptide chains with high amino acid sequence homology (93% identity in A-chain and 100% identity in B-chain). As shown in Fig. 1, ACF II has a typical structure of IX/X-bp (PDB code 1Y17). It is a heterodimer protein consisting two homologous chains (A-chain: 129 residues and B-chain: 123 residues) with a similar topology structure linked with a disulfide-bond. Each chain has one Ca^{2+}-binding site. Ca^{2+} ions have been found to not only increase the structural stability of ACF II but also induce the binding of ACF II with FXa [10]. ACF II contains nice residues in A-chain that are different from those of ACF I. The structure of ACF I (PDB code 1WT9) is very similar to that of ACF II.

Abbreviations: ACF I, anticoagulation factor I; ACF II, anticoagulation factor II; FIX, coagulation factor IX; FIXa, activated coagulation factor IX; FX, coagulation factor X; FXa, activated coagulation factor X; IX/X-bp, coagulation factor IX/coagulation factor X-binding protein; habu IX/X-bp, habu coagulation factor IX/factor X-binding protein; habu IX-bp, habu coagulation factor IX-binding protein; Deinagkistrodon X-bp, *Deinagkistrodon* coagulation factor X-binding protein; SNP, sodium nitroprusside dihydrate; L-NAME, N-omega-L-arginine methyl ester; PPT, plasma prothrombin time; APTT, activated partial thromboplastin time; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; nNOS, neuronal NO synthase; PE, phenylephrine; BP, blood pressure; MABP, mean arterial blood pressure; HR, heart rate.

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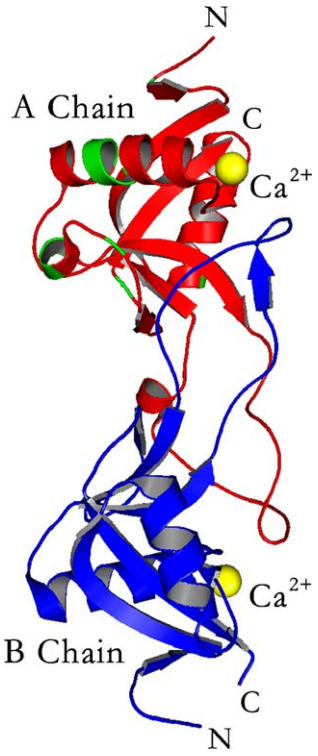

Fig. 1. Ribbon model of the heterodimer polypeptide chains of anticoagulation factor II (ACF II). The Ca^{2+} ions are indicated as yellow balls. The residues S5, A16, A24, A52-H53, K108-A109, R112 and R122 in A-chain of ACF II that are different from those of ACF I are marked in green. The picture was drawn with Protein Data Bank file 1Y17. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Despite the structural and anticoagulation mechanism studies have been reported for several IX/X-bps, their cardiovascular functions remain unclear. In this paper, the hypotensive effect of ACF I and ACF II in normotensive rats and L-NAME-induced hypertensive rats are described. Interestingly, the present results indicate that ACF II is not only able to block the amplification of the coagulation cascade by binding with FXa, but also able to cause a nitric oxide-dependent hypotensive effect in rats. ACF II has so far been identified as the first unique bifunctional protein in the IX/X-bp family that has both anticoagulant and hypotensive effects on the blood of rats through different pathways. However, ACF I does not show any hypotensive effect in rats.

Many small molecules and short peptides have hypotensive effects via various pathways [11–14], however, only a few natural proteins have been reported to possess hypotensive activity in vivo [15,16]. The natural proteins with both anticoagulant and hypotensive effects are quite rare. The discovery of the hypotensive effect of ACF II should be helpful to better understand its function as well as to develop bifunctional antithrombotic drugs with both anticoagulant and hypotensive activities.

2. Materials and methods

2.1. Materials

Male Wistar-Imamichi rats (180–250 g, 7–8 weeks old, supplied by Animal Services Center of Anhui Medical University, China) were used in all experiments. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication 85-23, revised 1996). Lyophilized venom powder was provided by the TUN-XI Snakebite Institute (Anhui, P. R. China). Activated bovine coagulation factor X (FXa) was a generous gift from Dr Zhao Chao, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Bovine serum albumin, acetylcholine hydrochloride, phenylephrine hydrochloride, sodium nitroprusside dihydrate (SNP) and N-omega-L-arginine methyl ester (L-NAME) were obtained from Sigma–Aldrich (St Louis, MO, USA). All other reagents were of analytical reagent grade and produced by the Shanghai Institute of Biochemistry (Shanghai, P.R. China).

2.2. Purification of ACF I and ACF II

ACF I and ACF II were purified by a three-step chromatography procedure of anion-exchange chromatography, gel permeation chromatography and cation-exchange chromatography as described previously [8]. The concentrations of ACF I and ACF II were calculated from their absorption coefficient ($A^{1\%}_{1\,cm} = 31$ for ACF I or 30 for ACF II) at 280 nm and their relative molecular weights (Mr = 29.6 kDa for ACF I or 29.5 kDa for ACF II). For in vivo experiments, all drugs were dissolved in physiological salt solution for administration at a volume of 1 ml/kg. The molecular homogeneity of the purified ACF I and ACF II was identified by polyacrylamide gel electrophoresis (native-PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [17].

2.3. Surface plasmon resonance (SPR)

SPR measurements were performed at 25 °C using a Biacore 3000. Sensor surfaces were pretreated and then normalized by standard Biacore protocols [18]. FXa was diluted in immobilization buffer (1 μM, in 10 mM sodium acetate, pH 5.0) and immobilized onto one flow cell of a CM5 chip. For coupling of FXa to CM5 sensor surfaces, the surfaces were activated with 70 μl of a mixture of 50 mM *N*-hydroxysuccinimide and 200 mM 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide. The activated chip was coated with 20 μg/ml FXa, dissolved in 10 mM sodium acetate buffer (pH 5.0), by injecting 60 μl of the solution at a flow rate of 10 μl/min. The remaining unchanged functional groups on the FXa-coated surface were blocked with 1 M ethanolamine hydrochloride, at a flow rate of 10 μl/min for 7 min. ACF I or ACF II (2 μM) in 0.02 M PBS buffer (pH 7.4) containing 150 mM NaCl and 1 mM Ca^{2+} was injected over the surface (10 μl/min, 20 μl injection with 300 s wash delay). The surface was regenerated between analyte injections with 1 M NaCl and 1 mM EDTA (50 μl at 20 μl/min).

2.4. In vivo anticoagulation measurements

To determine the effect of ACF II on coagulation after i.v. administration, rats were anaesthetized using a mixture of 25% urethane and 1% α-chloralose (w/v) given intraperitoneally at a dose of 5 ml/kg body weight. ACF II was administered through the femoral vein [19]. Ten min after administration of ACF II, 2 ml of arterial blood was drawn from the abdominal aorta into 3.8% citrate solution immediately and centrifuged. The plasma was collected for analysis of activated partial thromboplastin time (APTT). APTT was monitored by an Automated Coagulation Analyzer (Sysmex CA-1500, America Dade) [20].

2.5. In vivo cardiovascular measurements

Rats were anaesthetized using a mixture of 25% urethane and 1% α-chloralose (w/v) given intraperitoneally at a dose of 5 ml/kg body weight. Supplemental doses were given through a jugular intravenous catheter whenever needed. The trachea was

cannulated to keep the respiratory passage patent. The femoral artery and vein were cannulated for the measurement of blood pressure and administration of ACF I or ACF II, respectively [21]. Immediately after the cannulation of the femoral artery, 0.2 ml of heparinized saline (125 IU/ml) was injected into it to prevent intravascular coagulation. The arterial cannula was coupled to a pressure transducer (Statham P23D, USA) which had been previously calibrated. This was in turn connected to a model MP100A Grass polygraph (BIOPAC systems, Santa Barbara, California, USA) for the recording of blood pressure (BP) and HR. The animals were allowed about 30 min for the stabilization of the BP before starting the experiments. The initial BP and HR were taken and then ACF I or ACF II was administered through the femoral vein. For the experiments in the presence of L-NAME, according to the method of Rees et al. [22,23], L-NAME (50 mg/kg) was administered through the femoral vein. L-NAME markedly increased the BP of the rats and slightly decreased the HR of the rats. About two min after administration the values of the BP and HR reached a plateau. Up to 10 min after the administration of L-NAME, ACF II was administered through the femoral vein. One rat received one dose of protein.

2.6. In vitro experiment

Rats were killed and the thoracic aortae were rapidly and carefully dissected and placed into ice-cold Krebs solution (pH 7.4) containing (in mM) 118 NaCl, 4.7 KCl, 1.1 $MgSO_4$, 1.2 KH_2PO_4, 1.5 $CaCl_2$, 25 $NaHCO_3$, and 10 Glucose. The aortae were carefully cleaned of adhering connective tissue and fat, and then cut into rings with a width of approximately 3 mm. All dissecting procedures were done with extreme care to protect the endothelium from inadvertent damage. In some aortic rings, the endothelial layer was mechanically removed by gently rubbing the luminal surface of the aortic ring back and forth several times with plastic tube. The aortic rings were suspended, by means of two L-shaped stainless-steel wires inserted into the lumen, in a tissue bath containing Krebs solution (pH 7.4) at 37 °C. 95% O_2 to 5% CO_2 was continuously bubbled through the bath [21]. The baseline load placed on the aortic rings was 1.0 g. Changes in isometric tension were recorded using a force-displacement transducer (JH-2, China) connected to a Bioelectric Signal Processing system (SKY-A8, China). The vascular endothelium integrity was assessed by verifying the contracted rings 70% relaxation while being treated with acetylcholine (ACh) (1 μM). The endothelium absence was confirmed when the relaxation by acetylcholine was less than 10%. The rings were then exposed to various drugs for 30 min and aortic relaxation was carried out by the cumulative addition of ACF II. At the end of experiment, SNP (100 μM) was applied to produce maximum relaxation, which was taken as 100%. After each test, the aortic rings were washed three times with fresh Krebs solution and allowed to equilibrate for 30 min [24,25]. The segments of aorta for ACF II and acetylcholine experiments were from the same rat.

2.7. Data analysis

All in vivo data (BP and HR) were monitored and recorded on a polygraph system (BIOPAC systems, USA), and these parameters were analyzed offline using Datview software (AcqKnowledge), allowing the analyst to select and average data at chosen intervals during the experiment. Data are reported as mean ± standard error of mean (S.E.M.). Student's paired or unpaired *t*-test, as appropriate, was used to determine the significance of differences between means and $P < 0.05$ was taken as statistically significant.

3. Results

3.1. Binding of FXa with ACF II assessed by SPR spectroscopy

ACF I and ACF II have been purified by a three-step chromatography procedure of DEAE-Sephadex A-50 anion-exchange chromatography, Sephadex G-75 gel permeation chromatography and CM-Sephadex C-50 cation-exchange chromatography. As shown in Fig. 2, both ACF I and ACF II give a single band on native-PAGE and SDS-PAGE, respectively, indicating that the purified ACF I and ACF II are fairly homogeneous. Our recent studies showed that the values of association constant (K_A) and dissociation constant (K_D) for the binding of ACF I with FXa were $(1.9 \pm 0.4) \times 10^6\ M^{-1}$ and $(5.3 \pm 1.2) \times 10^{-7}$ M, respectively, determined by SPR spectroscopy [26]. In order to determine the binding affinity of FXa for ACF II, SPR spectroscopy was used to monitor the interaction of ACF II with FXa. Fig. 3 illustrates association and dissociation curves for ACF II interacting with FXa in the presence of 1 mM Ca^{2+}. Like ACF I, ACF II shows specific binding with FXa in the presence of 1 mM Ca^{2+}. The kinetic parameters, on-rate (k_{on}), off-rate (k_{off}), K_A and K_D have been obtained to be $(4.4 \pm 0.3) \times 10^4\ M^{-1}\ s^{-1}$, $(2.7 \pm 0.5) \times 10^{-2}\ s^{-1}$, $(1.7 \pm 0.2) \times 10^6\ M^{-1}$ and $(6.0 \pm 0.9) \times 10^{-7}$ M, respectively, by fit of the data to a 1:1 Langmuir model. The values of K_A and K_D for the binding of ACF II with FXa are very similar to those for the binding of ACF I with FXa, respectively, indicating that both ACF I and ACF II have a similar binding affinity for FXa.

3.2. Hypotensive effect of ACF II on anaesthetized normotensive rats

In order to investigate whether ACF I and ACF II have cardiovascular effects, the hypotensive activity of ACF I and ACF II has been measured in normotensive rats. Bovine serum albumin (BSA) has been chosen as the control. Fig. 4 shows the effects of ACF I (8 mg/kg iv), ACF II (4–8 mg/kg iv) and BSA (8 mg/kg iv) on the HR and MABP of normotensive rats. Baseline values of MABP are 96.6 ± 4.8 mmHg, and baseline values of HR are 360 ± 14 beats/min. As shown in Fig. 4A, BSA slightly increases both MABP and HR, as reported by Hayashida et al. [16]. The maximum increases in MABP (8.1 ± 0.3%) and HR (4.0 ± 0.1%) have been observed at about 100 s after the administration of BSA at a dose of 8 mg/kg and then the values of MABP or HR reach a plateau. ACF I does not show obvious effect on both MABP and HR of rats at a dose of 8 mg/kg (Fig. 4B).

Interestingly, ACF II causes a dose-dependent decrease of blood pressure (Fig. 4C). At the low doses of 4–5 mg/kg, ACF II causes an obvious decrease of MABP, and the maximum hypotensive effect of ACF II has been observed at about 5 s after the administration, then the MABP shows a recovery. After about 1–2 min, the values of MABP reach a plateau that is slight below original baseline. When ACF II was given at the high doses of 6–8 mg/kg, the maximum hypotensive effect of ACF II has been observed at about 30–50 s after the administration, then the MABP shows a recovery. After about 3–6 min, the values of MABP reach a plateau that is obviously below the baseline and lasts more than 2 h until the end of the experiment. The maximum changes in systolic BP, diastolic BP and MABP by BSA or ACF II are shown together in Fig. 4D. A maximum decrease of 54.9 ± 3.3% of MABP from 96.8 ± 5.7 to 43.7 ± 4.9 mmHg has been observed after the injection of ACF II at a dose of 7 mg/kg (Fig. 4C and D). At the dose of higher than 8 mg/kg, the BP of the rats treated was unstable and the significant drop of MABP might cause the rats to die. Based on our results, an ACF II dose of 7 mg/kg was chosen for the subsequent experiments. These results indicate that ACF II induces a rapid drop of MABP followed by an increase and then a longer lasting slight decrease in MABP. Fig. 4C shows that the ACF II-induced longer lasting slight depression of MABP is also in dose-dependence. Therefore, ACF II is a bifunctional protein with both anticoagulant and hypotensive activities. However,

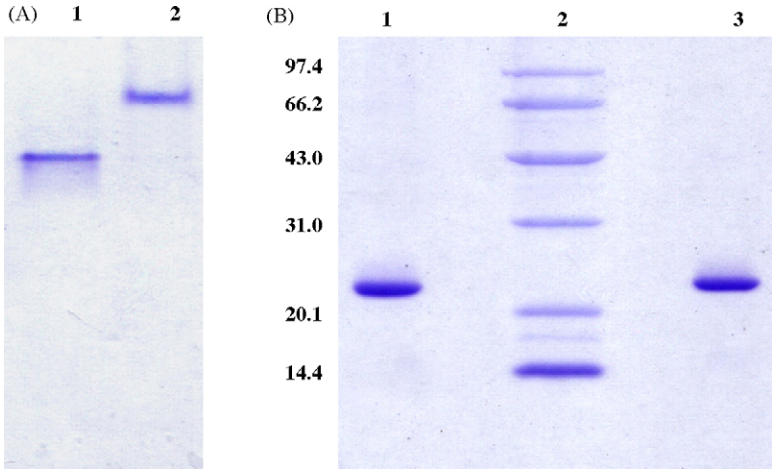

Fig. 2. Native-PAGE and SDS-PAGE of ACF I and ACF II. Purified ACF I and ACF II were electrophoresed in 12% polyacrylamide gels without SDS for native-PAGE (A) and containing 0.1% SDS under nonreducing condition for SDS-PAGE (B), respectively. (A) Lane 1, ACF I; lane 2, ACF II. (B) Lane 1, ACF I; lane 2, molecular mass markers; lane 3, ACF II.

no significant changes in the HR have been found throughout the experiment at all doses of ACF II (4–8 mg/kg) (Fig. 4E).

3.3. The mediation of the hypotensive effect of ACF II by NO production

NO is known to be involved in the central regulation of the cardiovascular functions, for example, NO is an endothelium-derived relaxing factor that causes vasodilation and controls vascular tone [16]. NO is synthesized from one of the terminal guanidino nitrogen atoms of the semi-essential amino acid L-arginine catalyzed by NO synthase [27]. L-NAME, a NO synthase inhibitor has been used to investigate whether NO release mediates the hypotensive effects of ACF II. According to the method of Rees et al. [16,22,23], the time courses of the changes in MABP in response to intravenous injection of ACF II (7 mg/kg) were measured in rats which had been pretreated intravenously 10 min earlier with L-NAME (50 mg/kg). Fig. 5 shows the influence of L-NAME on the hypotensive effects of ACF II. The NO synthase inhibitor, L-NAME markedly increases MABP and slightly decreases HR (Fig. 5A), as reported by Hayashida et al. [16]. Baseline value of MABP increases from 96.6 ± 4.8 mmHg to 157.1 ± 5.0 mmHg, in the presence of L-NAME (50 mg/kg). After pretreatment with L-NAME (50 mg/kg), the decrease in MABP in response to ACF II is significantly reduced (Fig. 5B and C). As shown in Fig. 5D, the ACF II-induced depressor response is significantly prevented by L-NAME in the whole depressor course. These results indicate that ACF II-induced hypotension in vivo is based on the ACF II-mediated release of NO.

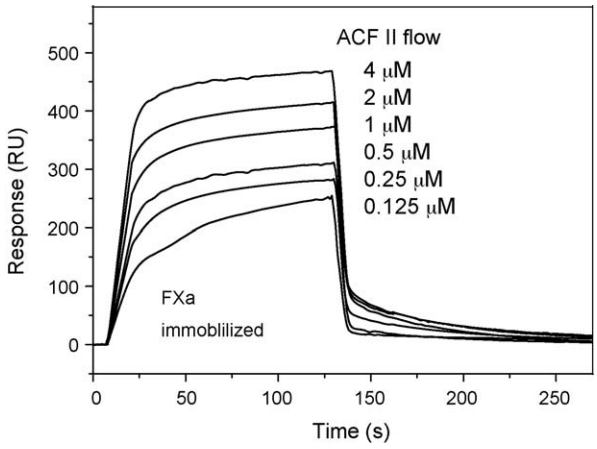

Fig. 3. SPR kinetic analysis of the interaction of ACF II with FXa. 20 μl aliquot of ACF II in 0.02 M PBS buffer (pH 7.4) containing 1 mM Ca^{2+} and 150 mM NaCl was injected over an immobilized FXa surface at concentrations of 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 μM at a flow rate of 10 μl/min for 2 min, and dissociation was monitored for 3 min. The kinetic parameters were obtained from the interaction by fit of the data to a 1:1 Langmuir model.

3.4. Effects of ACF II on phenylephrine-precontracted rat aortic rings

Vascular endothelial cells synthesize NO from L-arginine. This synthesis of NO accounts for the biological actions of endothelium-derived relaxing factor on vascular strips [22]. In order to examine whether ACF II induces vasodilation, the vascular responses to ACF II have been determined in the endothelium-intact and -denuded aortic rings. The isolated thoracic aorta rings have been precontracted by 1 μM phenylephrine (PE) to produce a sustained contractile tone over the course of the experiment. As shown in Fig. 6A, in phenylephrine-precontracted rat thoracic aorta rings with endothelium, ACF II indeed induces a concentration-dependent relaxation and cumulative dosing of ACF II results in an EC_{50} value of 30.0 ± 9.4 μM. When endothelium is removed, ACF II (up to 30 μM) is unable to relax phenylephrine-precontracted aortic rings (Fig. 6B and C). At the end of each experiment, SNP (100 μM) produces a maximum relaxation. These results reveal that ACF II is able to induce an endothelium-dependent vasodilatation that leads to a decrease in MABP.

To demonstrate whether the ACF II-mediated relaxation is based on the release of NO from endothelium, the relaxant effect of ACF II has been investigated in the presence of L-NAME (100 μM). As shown in Fig. 7, when the thoracic aorta rings are pretreated with 100 μM L-NAME, relaxation induced by 30 μM ACF II is inhibited by 56%. ACh induces vasodilatation via the NO pathway. 100 μM L-NAME inhibits ACh (5 μM)-induced relaxation by 92%, as reported by other authors [22]. This result shows that the release of NO from endothelium is involved in ACF II-induced relaxation.

3.5. Effect of ACF II on APTT in vivo

ACF II shows marked anticoagulant activity in vitro [8]. The anticoagulant effects of ACF II in vivo have been measured by APTT assay. The APTT of rats is 18.4 ± 1.4 s for the saline group (control). As shown in Fig. 8, intravenous administration of ACF II prolongs APTT in a dose-dependent manner. APTT is prolonged to more than six-fold by ACF II at a dose of 5 mg/kg. This result indicates that ACF II prolongs the APTT in vivo by binding to FXa.

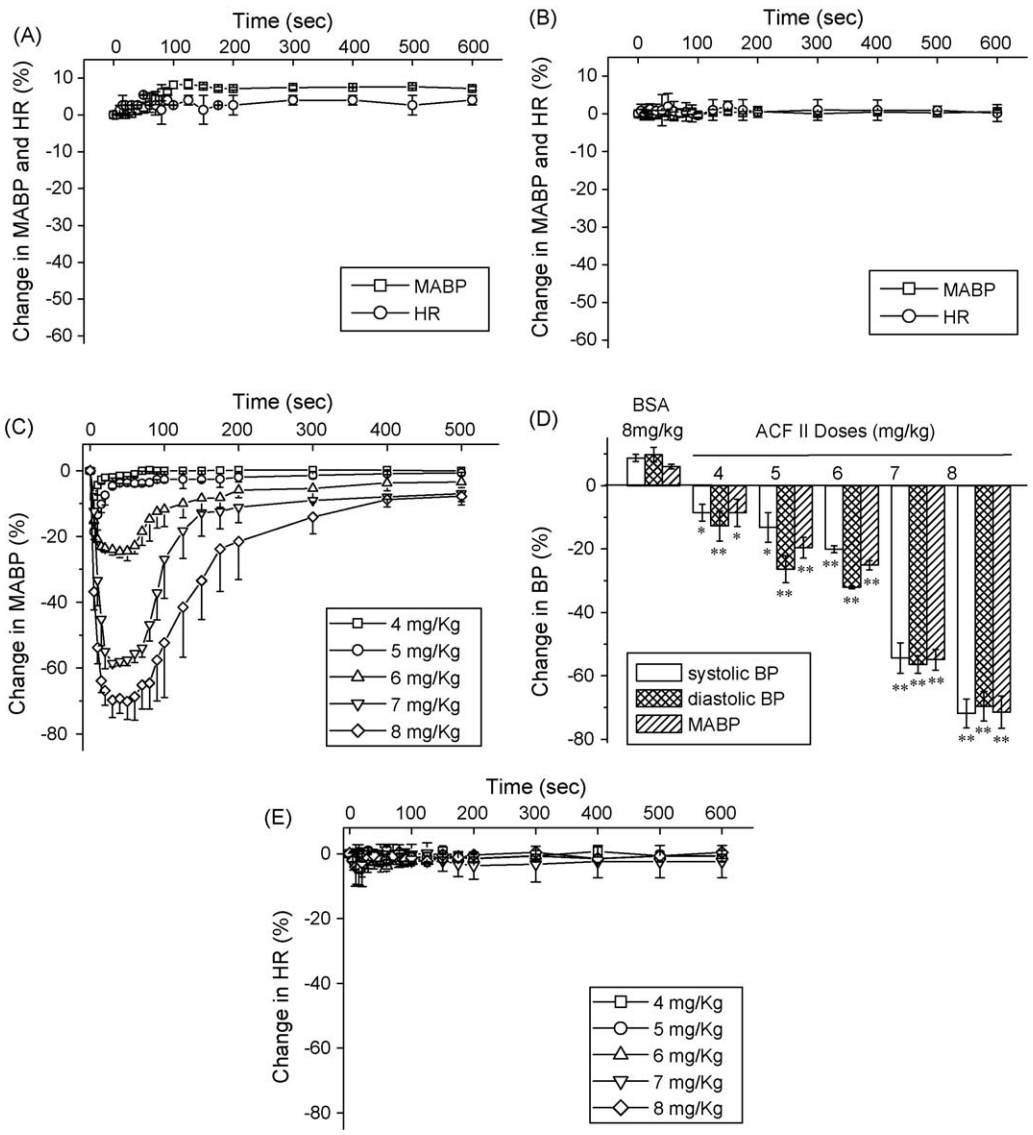

Fig. 4. Effect of ACF I and ACF II on arterial blood pressure and heart rate of rats. Time courses of the changes in MABP and HR in response to intravenous injection of BSA (8 mg/kg) (A) and ACF I (8 mg/kg) (B). Time courses of the changes in MABP (C) and HR (E) in response to intravenous injection of ACF II (4–8 mg/kg). (D) Maximum changes in BP in response to the intravenous injection of BSA (8 mg/kg) and ACF II (5–8 mg/kg). Means ± S.E.M., $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. BSA-treated group (Dunnett's test).

4. Discussion

Previously, we have identified ACF II as a FXa-binding protein with marked anticoagulant activity [9]. In the present study, one of the most intriguing observations is that ACF II is not only able to bind with FXa to block the amplification of the coagulation cascade, but also able to induce a hypotensive effect in rats. To our knowledge, in the family of IX/X-bps, only ACF II possesses this hypotensive activity. Therefore, ACF II is so far identified as the first unique bifunctional protein in the IX/X-bp family with both anticoagulant and hypotensive activities. Interestingly, both anticoagulation and hypotension induced by ACF II are in association with blood. ACF II is devoid of hemorrhagic activity [8]. We did not observe any major or minor bleeding in vivo experiments. ACF II may provide a useful clue for designing bifunctional antithrombotic drugs.

ACF I, another FXa-binding protein from the venom of *A. acutus* [8], has a similar binding affinity for FXa and a similar structure to that of ACF II. However, in spite of the high degree of structural similarity between the two proteins, ACF I does not show any hypotensive effect in rats (Fig. 4B). This result suggests that FXa binding is not involved in ACF II-induced vasodilatation.

As shown in Fig. 4C, intravenous injection of ACF II causes a dose-dependent decrease of MABP. ACF II induces a short fast drop of MABP followed by an increase and then a longer lasting slight decrease in MABP. Both fast decrease and long lasting slight decrease in MABP induced by ACF II are in dose-dependence. The recovery of MABP to the initial level is due to the compensatory sympathetic nerve reflex. This compensatory sympathetic nerve reflex may cause tachycardia. For example, hydralazine can reflexibly increase HR when it relaxes the smooth muscle of blood vessel to induce hypotension. On the other hand, Morphine, an opioid agonist can decrease both HR and MABP at the same time [16]. It is very interesting that, ACF II does not obviously affect HR in the process of decreasing MABP (Fig. 4E), suggesting that the effect of ACF II on the cardiovascular system is different from that of morphine or hydralazine. The slight decrease in MABP can last for at least 2 h, indicating that ACF II has a long hypotensive effect in rats.

It has been reported that thrombin receptor activating peptide (TRAP) also induces a relaxant response in rats similar to that

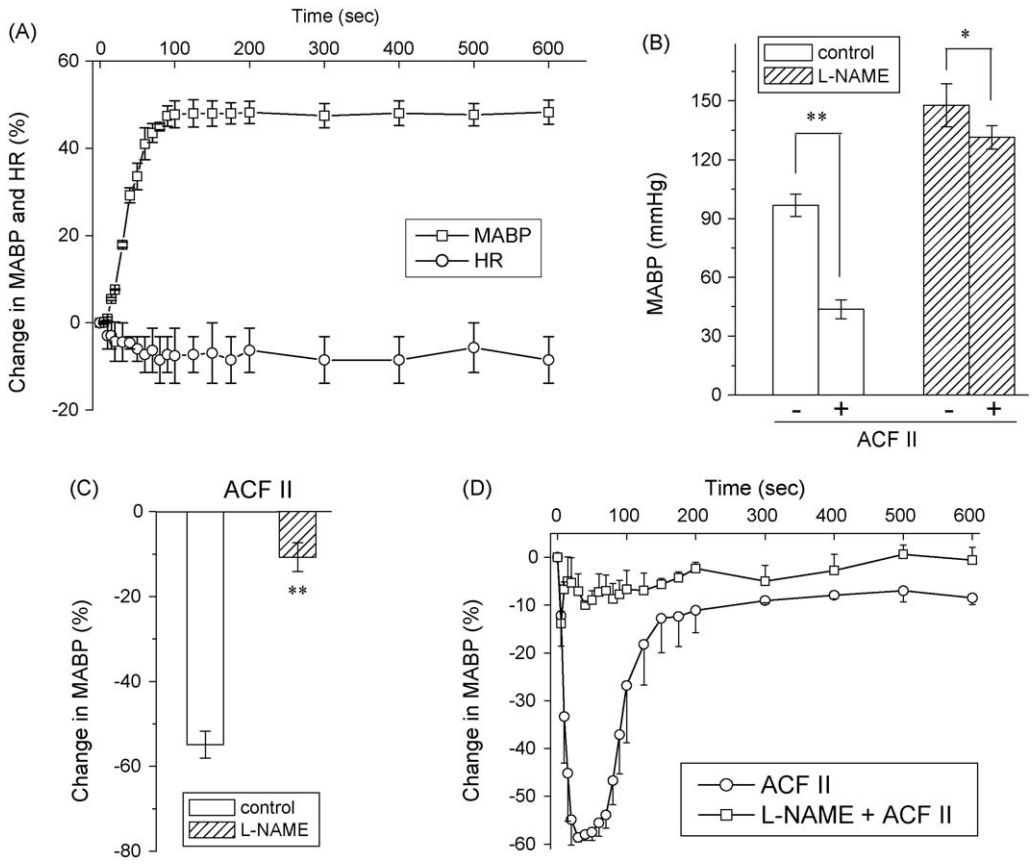

Fig. 5. Influence of nitric oxide (NO) synthase inhibitor on the hypotensive effect of ACF II. (A) Time courses of the changes in MABP and HR in response to intravenous injection of L-NAME (50 mg/kg). Actual values in MABP (B) and maximum changes in MABP (C) in response to the intravenous injection of ACF II (7 mg/kg) were measured in the presence or absence of N-omega-L-arginine methyl ester (L-NAME; 50 mg/kg iv). (D) Time courses of the changes in MABP in response to intravenous injection of ACF II (7 mg/kg) in the presence or absence of L-NAME (50 mg/kg iv). Means ± S.E.M., $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (Dunnett's test).

caused by ACF II [28]. TRAP-induced reaction on MABP is in part due to the release of endothelial NO. NO is known as an endothelium-derived relaxing factor that causes vasodilation and controls vascular tone [27]. Therefore, we have investigated whether NO is involved in ACF II-induced hypotension. As shown in Fig. 5, ACF II-induced hypotension is significantly abolished by L-NAME, a nitric oxide synthase inhibitor, in vivo, suggesting that NO plays a key role in ACF II-induced hypotension.

In vitro experiment (Fig. 6), ACF II exerts a concentration-dependent vasorelaxant effect on phenylephrine-contracted aortic rings from rats. Removing functional-endothelium completely abolishes the relaxant response to ACF II, suggesting that the vasorelaxation caused by ACF II is endothelium-dependent. To determine whether NO is involved in ACF II-induced vasorelaxation, the effect of L-NAME on ACF II-induced vascular relaxation has been examined. Pretreatment of aortic tissues with 100 μM L-NAME, markedly abolishes the ACF II-induced vascular relaxation, indicating that NO is likely to be a mediator of this effect. These results together with observations in vivo experiment suggest that the ACF II-induced hypotension is closely related with the activation of a NO pathway.

ACh induces vasodilatation via the NO pathway. It is notable that while L-NAME greatly attenuates the relaxant response to ACh in vitro, L-NAME has a lesser effect on the relaxant response to ACF II (Fig. 7). This result suggests that the ACF II-induced hypotension is only in part related with the activation of a NO pathway. Another unidentified pathway seems to be also involved in ACF II-induced hypotension.

In comparison to a lot of small molecules with hypotensive activity, only a few natural proteins have been reported to possess hypotensive activity in vivo. Interestingly, most natural proteins with hypotensive activity are multifunctional proteins. For example, five serine proteases have been identified with hypotensive effect in vivo, including thrombin [28,29], trypsin [30], plasmin [31], neutrophil elastase and chymotrypsin [32]. These five proteases are all multifunctional enzymes [33–37]. Thrombin is a well-known multifunctional protein that induces a series of receptor-mediated events in various cell types, such as endothelial cells, smooth muscle cells and platelets [38,39]. Currently, only one nonenzymatic protein has been documented with hypotensive effect in vivo, which is bovine lactoferrin [16]. Lactoferrin is also a multifunctional protein that is found in milk, neutrophils, and other biological fluids. Lactoferrin is an iron-binding glycoprotein, which consists of a single chain polypeptide with 689 residues [40]. It is well known for its function in iron transport. In addition, lactoferrin induces hypotension via an endothelium-dependent vasodilation, which is strongly mediated by NO production. It is reported that the blood pressure-buffering effect of endogenous NO is mediated by endothelial NO synthase (eNOS) [41]. Lactoferrin was supposed to activate eNOS via its receptor-mediated mechanism or via direct interaction with eNOS, which resulted in a decrease in MABP [16]. Another observation suggested that bovine lactoferrin selectively activated neuronal NO synthase (nNOS) to accelerate NO production [42].

Present study shows that ACF II is the second nonenzymatic protein with hypotensive effect in vivo. Like lactoferrin, ACF II induces hypotension via an endothelium-dependent vasodilation, which is also strongly mediated by NO production. Interestingly enough, both ACF II from snake venom and lactoferrin from milk have a hypotensive effect in rats. ACF II causes a decrease of MABP

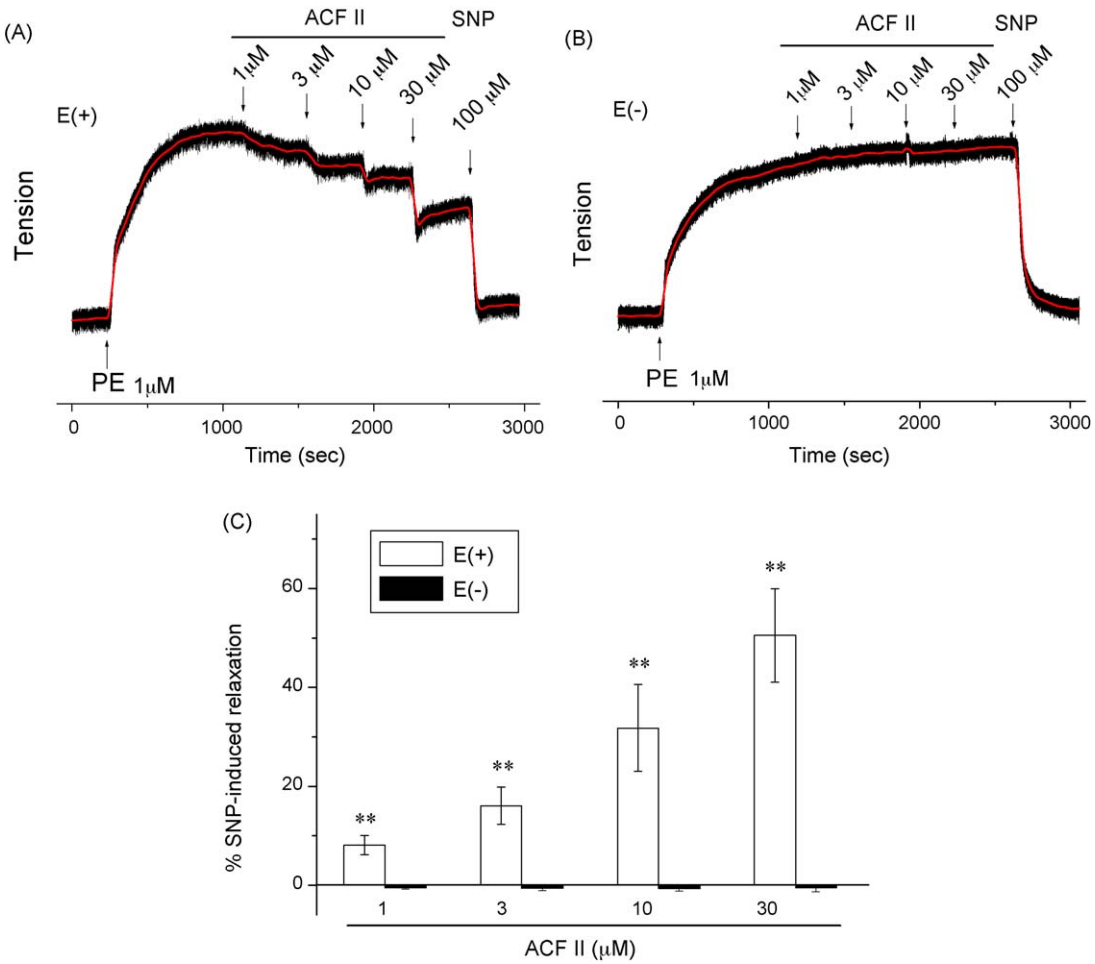

Fig. 6. ACF II-induced relaxation in the rat thoracic aorta. The typical responses to ACF II were determined in endothelium-intact (E+) (A) and endothelium-denuded (E−) (B) aortic rings precontracted with phenylephrine (PE), respectively. (C) Data are expressed in terms of relative relaxation using the maximum relaxation induced by sodium nitroprusside (SNP) as references (100%). Means ± S.E.M., $n = 8$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. endothelium-denuded group (unpaired t-test).

(−54.9 ± 3.3%) at a dose of 7 mg/kg, namely 237 nmol/kg, while lactoferrin causes a decrease of MABP (−35%) at a dose of 1280 nmol/kg [16], indicating that ACF II is more effective than lactoferrin as a vasodepressor.

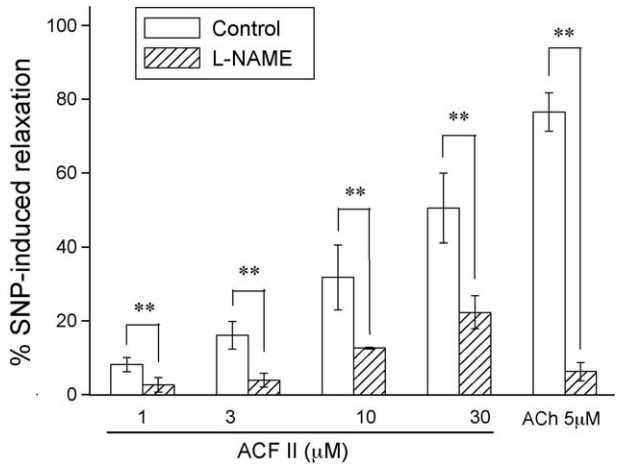

Fig. 7. Effect of the NO synthase inhibitor on ACF II-induced relaxation in the rat thoracic aorta. The responses of endothelium-intact aortic rings to ACF II were determined in the presence or absence of L-NAME (100 μM). The segments of aorta for ACF II and acetylcholine experiments were from the same rat. Means ± S.E.M., $n = 8$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (Dunnett's test).

In contrast to anticoagulant enzymes that convert coagulation factors into degraded forms and prolong the clotting time, ACF II, as a nonenzymatic anticoagulant, has a unique anticoagulant mechanism, for it forms a 1:1 complex with FXa and thereby blocks the amplification of the coagulation cascade [9]. ACF II can produce relaxation in endothelium-intact aortic rings

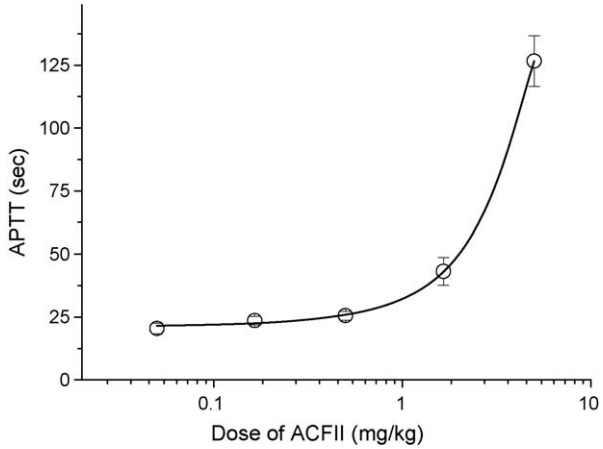

Fig. 8. Effect of ACF II on APTT in vivo after i.v. administration of ACF II in anaesthetized rats. ACF II was administered through the femoral vein in anaesthetized rats at selected doses. Blood samples were taken out 10 min after administration of ACF II, and then APTT was measured. Means ± S.E.M., $n = 5$.

precontracted with phenylephrine in FXa-free Krebs solution, suggesting that FXa is not involved in ACF II-induced vasodilation and that ACF II shows its anticoagulant and hypotensive activities via different pathways. As shown in Fig. 8, the minimum dose of ACF II to prolong APTT is about 1.0 mg/kg. The minimum dose of ACF II needed for the hypotension in rats is about 4 mg/kg. Therefore, the anticoagulant and hypotensive effects of ACF II occur at different concentrations. This result further suggests that the anticoagulation and hypotension of ACF II occur via different pathways. ACF II contains nice residues in A-chain that are different from those of ACF I (Fig. 1). Some of the nice residues should be responsible for the hypotensive activity of ACF II. Although we cannot infer the detailed picture of the pathway of ACF II-induced hypotension from present data, it is certain that ACF II is bifunctional protein that has two different effects on the blood through different pathways. Further investigation is necessary to clarify the detailed mechanism of the hypotensive activity of ACF II.

In conclusion, the present study reveals that ACF II induces a dose-dependent response in rats with a short fast drop of MABP followed by an increase and then a longer lasting slight decrease in MABP, but does not obviously affect HR. ACF II-induced hypotension is significantly blocked by L-NAME. ACF II produces a concentration-dependent relaxation of rat aortic rings with functional-endothelium. The vasodilatation caused by ACF II is completely inhibited by removal of endothelium and significantly inhibited by pretreatment with L-NAME. These results demonstrate that ACF II induces hypotension through an endothelium-dependent vasodilation, which is strongly mediated by the release of NO from endothelium. ACF II exhibits high anticoagulation activity in vivo based on APTT assay. Therefore, ACF II is so far identified as the first unique bifunctional protein in the IX/X-bp family that has both anticoagulant and hypotensive effects on the blood of rats through different pathways.

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