Antithrombotic Effect of Crotalin, a Platelet Membrane Glycoprotein Ib Antagonist From Venom of Crotalus atrox

By Mei-Chi Chang, Hui-Kuan Lin, Hui-Chin Peng, and Tur-Fu Huang

A potent platelet glycoprotein Ib (GPIb) antagonist, crotalin, with a molecular weight of 30 kDa was purified from the snake venom of Crotalus atrox. Crotalin specifically and dose-dependently inhibited aggregation of human washed platelets induced by ristocetin with IC_{50} of 2.4 μg/mL (83 nmol/L). It was also active in inhibiting ristocetin-induced platelet aggregation of platelet-rich plasma (IC_{50} 6.3 μg/mL). Furthermore, crotalin bound to human platelets in a saturable and dose-dependent manner with a k_d value of 3.2 ± 0.1 x 10^{-7} mol/L, and its binding site was estimated to be 58,632 ± 3,152 per platelet. Its binding was specifically inhibited by a monoclonal antibody, AP1 raised against platelet GPIb. Crotalin significantly prolonged the latent period in triggering platelet aggregation caused by low concentration of thrombin (0.03 U/mL), and inhibited thromboxane B_2 formation of platelets stimulated either by ristocetin plus von Willebrand factor (vWF), or by thrombin (0.03 U/mL). When crotalin was intravenously (IV) administered to mice at 100 to 300 μg/kg, a dose-dependent prolongation on tail bleeding time was observed. The duration of crotalin in prolonging tail bleeding time lasted for 4 hours as crotalin was given at 300 μg/kg. In addition, its in vivo antithrombotic activity was evidenced by prolonging the latent period in inducing platelet-rich thrombus formation by irradiating the mesenteric venules of the fluorescein sodium-treated mice. When administered IV at 100 to 300 μg/kg, crotalin dose-dependently prolonged the time lapse in inducing platelet-rich thrombus formation. In conclusion, crotalin specifically inhibited vWF-induced platelet agglutination in the presence of ristocetin because crotalin selectively bound to platelet surface receptor-glycoprotein Ib, resulting in the blockade of the interaction of vWF with platelet membrane GPIb. In addition, crotalin is a potent antithrombotic agent because it pronouncedly blocked platelet plug formation in vivo.

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Previously described methods with slight modification. Aliquot of Capintec, CRC-7, continuously.

Human platelet aggregation. Blood was collected from healthy human volunteers, who did not take any medication within the 2 weeks before the study, and anticoagulated with 3.8% sodium citrate (9:1, vol/vol). Citrated blood was immediately centrifuged for 10 minutes at 120g and 25°C, and the supernatant (platelet-rich plasma) was obtained. Human washed platelet suspension was prepared as previously described. Washed platelets were suspended in modified Tyrodes’ solution (pH 7.3) (in mmol/L: NaCl, 136.9; CaCl2, 2; KCl, 2.7; MgCl2, 1.0; NaH2PO4, 0.4; NaHCO3, 11.9; glucose, 11.1) containing bovine serum albumin (3.5 mg/mL) and adjusted to about 3 x 10^8 platelets/mL.

The turbidimetric method, using a Lumi-Aggregometer (Chrono-Log, Havertown, PA), was used to measure platelet aggregation. The extent of aggregation was expressed in light transmission unit.

Measurement of bleeding time in mice. Melting point of crotal venom through columns of DEAE-Sephadex A-50 and Sephadex G-75 and refractionation by fast protein liquid chromatography using Mono-S (Fig 1A) and Superoxide columns (Fig 1B). The purified fraction migrated as a single band and the apparent molecular weight was estimated to be 30 kD under nonreducing or reducing conditions by SDS-polyacrylamide (15%) gel electrophoresis (Fig 1B, inset) and named crotalin.

Amino acid analysis showed that crotalin is a polypeptide, consisting of about 260 amino acid residues (Asp/Asn 36, Glu/Gln 25, Ser 14, Gly 21, His 9, Thr 8, Ala 14, Arg 22, Pro 10, Tyr 6, Val 16, Ile 18, Leu 28, Cys 7, Phe 10, and Lys 16). However, the N-terminal amino acid sequence of crotalin was found to be blocked.

Inhibition of ristocetin-induced platelet aggregation. Crotalin showed a marked inhibitory effect on ristocetin (1.0 mg/mL)-induced human washed platelet aggregation with IC50 value of 2.4 µg/mL (Fig 2). This inhibitory effect was independent of the incubation time of crotalin with platelets. Furthermore, crotalin was specific for ristocetin-induced platelet aggregation because it had little effect on the platelet aggregations caused by ADP (20 µmol/L) plus 200 µg/mL of fibrinogen, U46619 (1 µmol/L), or collagen (10 µg/mL). Crotalin also inhibited ristocetin (1 mg/mL)-induced platelet aggregation of platelet-rich plasma with IC50 value of 6.3 µg/mL (Fig 2), indicating that it might have an antithrombotic effect in vivo.

Similarly, crotalin (10 µg/mL) apparently did not affect platelet aggregation caused by high concentration of thrombin (>0.05 U/mL). However, crotalin (20 to 100 µg/mL) prolonged the latent period in triggering platelet aggregation in a dose-dependent manner, with a slight inhibition on the maximal aggregation (<30%) inhibition. The MoAb AP1 (40 to 80 µg/mL) showed a similar effect to that of crotalin (data not shown).

Effect of crotalin on thromboxane B2 formation of platelets caused by thrombin and other agonists. As shown in Table 1, crotalin at a lower concentration (5 to 10 µg/mL) inhibited thromboxane B2 formation of platelets stimulated by ristocetin and vWF. At higher concentration (20 µg/mL), crotalin significantly inhibited thromboxane B2 formation of platelets caused
by thrombin (0.03 U/mL), whereas it did not affect thromboxane B2 formation stimulated by collagen.

Characterization of the binding of 125I-crotalin to human platelets. 125I-crotalin bound to platelets in a dose-dependent manner, reaching a saturated binding at 10 µg/mL (0.33 µmol/L) (Fig 3). The Scatchard analysis of 125I-crotalin binding data showed that the binding sites of crotalin were 58,632 ± 3,152 per platelet with a kd value of 3.2 ± 0.1 × 10^-7 mol/L (Fig 4). 125I-crotalin binding to platelets was blocked either by unlabeled crotalin (100 µg/mL, >85%) or AP1 (20 µg/mL, >90%), but not by the MoAb against GPIIb/IIIa, 7E3 (40 µg/mL), or 5 mmol/L EDTA (data not shown).

Effect of crotalin on bleeding time of mice. IV administration of crotalin to mice significantly prolonged the bleeding time in a dose-dependent manner (Fig 5). Crotalin pronouncedly prolonged the bleeding time (>10 minutes) as measured 1 hour after a bolus injection of crotalin (300 µg/kg), and this effect slowly ran down thereafter (Fig 6). The bleeding time almost returned to the baseline level within 4 hours after the administration of crotalin. However, the platelet count, as

![Graph A](image1.png)

![Graph B](image2.png)

Fig 1. (A) Rechromatography of crotalin on Mono-S column. The active fraction was dissolved in 0.02 N ammonium acetate, pH 5.0, and loaded on Mono-S column. Elution was carried out with a gradient from 0 to 0.75 mol/L NaCl as indicated (---) at a flow rate of 0.5 mL/min. The active fraction (*) was collected. (B) Gel filtration chromatography on Superose HR 10/30 column. The active fraction above was applied (5 mg) to this column equilibrated with 0.05 mol/L phosphate buffer (pH 7.2). The column was eluted at a flow rate of 0.25 mL/min with the same buffer. The absorbance profile monitored at 280 nm was shown. The active fraction (*) which was eluted at 70 minutes was named crotalin.
measured at 10 minutes after the administration of crotalin (300 µg/kg), did not show a major change, although about 20% decrease was observed (128 ± 10 × 10^4, n = 4, control vs. 104 ± 13 × 10^4 platelets/µL, n = 4, experimental; P > .05). Even when the dose of crotalin was increased to 600 µg/kg, a transient slight decrease of platelet count (about 20%) was observed at the first 5 minutes after the administration of crotalin, and the platelet count returned to control level within 20 minutes.

Platelet-rich thrombus formation in the microvessels. As the given dose of fluorescein sodium was increased, the latent period in inducing platelet plug formation was shortened (Table 2). IV administration of crotalin at 300 µg/kg pronouncedly delayed platelet-rich thrombus formation and significantly prolonged the occlusion time in mice receiving different doses of fluorescein sodium (Table 2). The occlusion time was lengthened from 100 ± 14 to 311 ± 25 seconds in fluorescein sodium (150 µg per mouse)-treated mice after IV administration of crotalin. Crotalin dose-dependently prolonged the occlusion time in causing platelet plug formation, and administration of 300 µg/kg of crotalin resulted in the maximal lengthening of occlusion time (Table 3). This antithrombotic effect lasted at least for 2 hours (Fig 7). On the other hand, a continuous infusion of prostaglandin I_2 (PGI_2) at 0.5 µg/kg/min, as in our previous study, showed a maximal lengthening effect on occlusion time. Higher dose (2 µg/kg/min) of PGI_2 did not...

**Table 1. Effects of Crotalin on Thromboxane B_2 Formation of Washed Platelets Stimulated by Thrombin and Other Agonist**

<table>
<thead>
<tr>
<th></th>
<th>Thromboxane B_2 (ng/mL)</th>
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<tbody>
<tr>
<td></td>
<td>Ristocetin + vWF</td>
</tr>
<tr>
<td>Control</td>
<td>12.4 ± 2.6</td>
</tr>
<tr>
<td>Crotalin</td>
<td></td>
</tr>
<tr>
<td>5 µg/mL</td>
<td>3.4 ± 0.6*</td>
</tr>
<tr>
<td>10 µg/mL</td>
<td>3.5 ± 0.4*</td>
</tr>
<tr>
<td>20 µg/mL</td>
<td>ND</td>
</tr>
<tr>
<td>40 µg/mL</td>
<td>ND</td>
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</tbody>
</table>

Human washed platelets were preincubated with saline or various concentrations of crotalin at 37°C for 5 minutes before the addition of ristocetin (1 mg/mL) plus vWF (10 µg/mL), thrombin (0.03 U/mL), and collagen (10 µg/mL). Six minutes after stimulation, the reaction was terminated by the addition of EDTA (2 mmol/L) and indomethacin (50 µmol/L). The basal value of resting platelets was 2.4 ± 0.5 ng/mL. Values are presented as mean ± SEM.

Abbreviation: ND, not determined.

* P < .01 as compared with the respective control, n = 5.
† P < .05.

**Fig 2.** Dose-response relationship of crotalin on platelet aggregation induced by 1 mg/mL of ristocetin in human washed platelets in the presence of 10 µg/mL of vWF (●) or in platelet-rich plasma (▲). Values are presented as mean ± SEM (n = 4).

**Fig 3.** Binding isotherm of 125I-crotalin on human platelet suspension. Platelets were incubated with various concentrations of 125I-crotalin. Total binding (●) and nonspecific binding (●●) in the presence of unlabeled crotalin (200 µg/mL) were determined, respectively. Specific binding (●●●) was calculated by subtracting the nonspecific binding from total binding. This is a representative one of four similar experiments.

**Fig 4.** Scatchard plot of the 125I-crotalin binding to human washed platelets. This plot is a representative one of four experiments.
further increase its antithrombotic activity. Halysin, an RGD-containing peptide purified from venom of A. halys, inhibited platelet aggregation via a competitive inhibition of fibrinogen binding to platelet GPIIb/IIIa. Halysin completely inhibited ex vivo platelet aggregation of platelet-rich plasma induced by collagen (15 µg/mL) 20 minutes after the IV administration of halysin at the dose of 10 mg/kg (data not shown). In comparing the maximal effect of PGI2, halysin, ancrod, and crotalin on the occlusion time in the same in vivo model, crotalin appears to be the most efficacious agent in prolonging the occlusion time (Table 4).

DISCUSSION

Crotalin, a newly purified protein from the venom of C. atrox, specifically inhibited ristocetin-induced platelet aggregation in vitro and exhibited the antithrombotic activity in vivo. Crotalin specifically inhibited platelet aggregation induced by ristocetin either in platelet suspension supplemented with vWF or in platelet-rich plasma with an IC50 of 2.4 and 6.3 µg/mL, respectively. In contrast to RGD-containing peptides, crotalin at 40 µg/mL did not affect collagen- and U46619-induced platelet aggregation. Ristocetin-induced platelet aggregation was mediated through the initial binding of vWF to platelet GPIb, and subsequently resulted in the exposure of the fibrinogen receptor. The 125I-crotalin binding site was 58,632 per platelet with a kd value of 3.2 × 10⁻⁷ mol/L. 125I-crotalin binding to platelets was selectively inhibited by AP1, an MoAb raised against platelet GPIb, but not by 7E3, an MoAb raised against GPIIb/IIIa. The binding was not affected by EDTA, indicating that the binding process is divalent-cation independent. Through the analysis of binding data and its inhibitory activity on ristocetin-induced platelet aggregation, crotalin appears to be a selective antagonist of platelet membrane GPIb. Regarding the binding sites of crotalin, the estimation of 58,632 per platelet is higher than that estimated with GPIb MoAb (around 30,000). It has been reported that the binding sites of other venom GPIb

Table 2. Effect of Fluorescein Sodium and Crotalin on the Occlusion Time in Causing Platelet-Rich Thrombus Formation in Mesenteric Venules of Mice

<table>
<thead>
<tr>
<th>Dose of Fluorescein Sodium per Mouse</th>
<th>Normal saline</th>
<th>Crotalin (300 µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µg</td>
<td>180 ± 23 (4)</td>
<td>310 ± 36 (4)*</td>
</tr>
<tr>
<td>150 µg</td>
<td>100 ± 14 (4)</td>
<td>311 ± 25 (5)*</td>
</tr>
<tr>
<td>200 µg</td>
<td>63 ± 7 (3)</td>
<td>155 ± 13 (3)*</td>
</tr>
</tbody>
</table>

Values are expressed as elapsed time (in seconds) in causing platelet plug formation on the irradiation of venules and presented as mean ± SEM (n).
*P < .001 as compared with the respective control.

Crotalin, a newly purified protein from the venom of C. atrox, specifically inhibited ristocetin-induced platelet aggregation in vitro and exhibited the antithrombotic activity in vivo. Crotalin specifically inhibited platelet aggregation induced by ristocetin either in platelet suspension supplemented with vWF or in platelet-rich plasma with an IC50 of 2.4 and 6.3 µg/mL, respectively. In contrast to RGD-containing peptides, crotalin at 40 µg/mL did not affect collagen- and U46619-induced platelet aggregation. Ristocetin-induced platelet aggregation was mediated through the initial binding of vWF to platelet GPIb, and subsequently resulted in the exposure of the fibrinogen receptor. The 125I-crotalin binding site was 58,632 per platelet with a kd value of 3.2 × 10⁻⁷ mol/L. 125I-crotalin binding to platelets was selectively inhibited by AP1, an MoAb raised against platelet GPIb, but not by 7E3, an MoAb raised against GPIIb/IIIa. The binding was not affected by EDTA, indicating that the binding process is divalent-cation independent. Through the analysis of binding data and its inhibitory activity on ristocetin-induced platelet aggregation, crotalin appears to be a selective antagonist of platelet membrane GPIb. Regarding the binding sites of crotalin, the estimation of 58,632 per platelet is higher than that estimated with GPIb MoAb (around 30,000). It has been reported that the binding sites of other venom GPIb
INTERACTION OF CROTALIN WITH GPIb

![Fig 7. The effects of crotalin on the elapsed time in causing platelet plug formation upon the irradiation of venules in mice. Fluorescein sodium (150 µg per mouse) was intravenously injected 10 minutes before the irradiation, and the irradiation was then started for inducing the formation of thrombus at the indicated time intervals after the IV administration of crotalin (300 µg/kg). Values are presented as mean ± SEM (n = 5-6). BL indicates baseline value. (>) Represents the occlusion time of each mouse measured at the indicated time. *P < .05, **P < .01 as compared with basal value.]

Platelet-rich thrombus formation was induced as described in Table 2. The occlusion time was measured 10 to 20 minutes after the administration of halysin, ancrod, crotalin, and PGI2. Values are presented as mean ± SEM (n = 6).

- Crotalin, 300 µg/kg: 293 ± 38 (6)
- Ancrod, 1 U/kg: 176 ± 31 (6)*†
- Halysin, 10 mg/kg: 155 ± 20 (6)*†
- PGI2, 2 µg/kg/min: 148 ± 16 (6)*†
- Saline: 105 ± 12 (6)

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This study, crotalin as well as API prolonged the latent period in triggering platelet aggregation caused by low concentrations of thrombin (0.03 U/mL), with a slight inhibition on platelet aggregation. Crotalin blocked thromboxane B2 formation of platelets challenged by ristocetin plus vWF or low concentration of thrombin, confirming the hypothesis that the ligation of GPIb may lead to the activation of endogenous phospholipase A2. Regarding the molecular characterization of crotalin, a platelet GPIb antagonist, the preliminary data show that crotalin is unique as a single chain polypeptide that is quite different from the known venom GPIb-binding proteins because they are heterodimer in nature, sharing a highly homologous sequence with C-type lectins. Whether it exists as a monomer or dimer under physiological condition is unknown. However, the detailed characterization of the physicochemical properties of crotalin is in progress.

An IV infusion of crotalin lengthened bleeding time of mice in a dose-dependent manner. Maximal prolongation was observed during a period of 10 to 60 minutes after injection of crotalin (300 µg/kg), and bleeding time progressively returned to control values over a 4-hour period. However, the platelet count after the administration of crotalin did not show a major change. It has been reported that echicetin and jararaca GPIb-BP caused a transient thrombocytopenia in mice, and therefore it may be an advantage of crotalin when considering its potential use as antithrombotic agent. From its in vivo experiment, we suggest that crotalin may inhibit platelet aggregation as well as platelet adhesion to subendothelium in vivo through blocking the interaction of vWF with platelet GPIb.

As the first step in hemostasis or thrombosis, the binding of vWF to platelet GPIb is essential for platelet adhesion at high-shear blood flow. The platelets from patients with Bernard-Soulier Syndrome were defective in expression of functional GPIb-IX complex, and poorly adhered to subendothelium at all shear rates. Much effort has recently been devoted to characterize the interaction of vWF and platelet GPIb at the molecular level with an aim of developing inhibitors that could be useful in the prevention of thrombosis. Recently, it has been indicated that inhibition of vWF-platelet GPIb interaction is effective in preventing acute restenosis after thrombolytic therapy.

In the present study we evaluated the antithrombotic effect of crotalin in a mouse model. Surprisingly, crotalin apparently delayed platelet-rich thrombus formation in mesenteric microvessels and its antithrombotic activity was dose dependent. The time course of its antithrombotic effect was consistent with that of its effect on bleeding time in mice (Figs 6 and 7).

Table 4 shows the minimal dose of PGI2, halysin, ancrod, and crotalin in causing the maximal prolongation of occlusion time in this platelet-rich thrombus animal model. Halysin, an RGD-containing venom peptide, completely inhibited ex vivo platelet aggregation for 20 minutes after the administration of halysin at 10 mg/kg. Ancrod (1 U/kg), a thrombin-like enzyme, caused defibrinogenation and exhibited antiplatelet activity for 60 minutes. Of the compounds tested, crotalin showed the most pronounced effect in prolonging the occlusion time of the irradiated vessels in inducing platelet-rich thrombus formation as compared with PGI2, halysin, and ancrod, indicating that blockade of the interaction between vWF and platelet GPIb may be a potential strategy in causing a marked antithrombotic
effect. In addition, crotalin exhibits an antithrombotic activity with a longer duration as compared with short duration of PGI₂, halysin or another disintegrin, triflavin.⁴⁰

Considering the pharmacokinetic of crotalin, crotalin may be more active as an antithrombotic agent in mice than in human beings because the effective dosage of crotalin in mice ranged from 100 to 300 µg/kg, equivalent to 1.3 to 3.8 µg/mL (assuming 2.0 mL plasma per mouse), even if protein binding is neglected. However, the IC₅₀ of crotalin was about 6.3 µg/mL (in human platelet-rich plasma), five times higher than the effective dosage of 100 µg/kg in mice. On the other hand, the in vivo antithrombotic effect of crotalin in mice may result from both the antiplatelet and anticoagulant activities because the preliminary results show that crotalin prolonged the whole blood clotting time and activated partial thromboplastin time but not the prothrombin time as crotalin was administered IV (unpublished data, December 1996). However, its anticoagulant activity is under investigation.

In conclusion, crotalin specifically inhibited ristocetin-induced platelet agglutination in the presence of vWF through a selective binding of platelet membrane GPIb, resulting in a blockade of interaction between vWF and GPIb. Furthermore, crotalin markedly prolonged the bleeding time when administered IV into mice and was efficacious in blocking platelet plug formation in vivo experimental model. Therefore, crotalin may be a valuable tool for developing a new class of antithrombotic drugs for clinic use through the study of its structure-activity relationship.

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