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ANTITHROMBOTIC PROPERTIES OF IXOLARIS, A POTENT INHIBITOR OF THE EXTRINSIC PATHWAY OF THE COAGULATION CASCADE

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Summary

Ixolaris is a two-Kunitz tick salivary gland protein identified in *Ixodes scapularis* that presents extensive sequence homology to TFPI. It binds to FXa or FX as scaffolds and inhibits extrinsic Xnase. Differently from TFPI, however, Ixolaris does not bind to the active site cleft of FXa. Instead, complex formation is mediated by the FXa heparin-binding exosite, which may also results in decreased FXa activity into the prothrombinase complex. In this report, we show that recombinant ¹²⁵I-Ixolaris interacts with rat and human FX in plasma and prolongs the prothrombin time (PT) and activated partial thromboplastin time (aPTT) *in vitro*. We have also investigated the effects of Ixolaris *in vivo*, using a venous thrombosis model. Subcutaneous (s.c.) or intravenous (i.v.) administration of Ixolaris in rats causes a dose-dependent reduction in thrombus formation, with complete inhibition attained at 20 µg/kg and 10 µg/kg, respectively. Remarkably, antithrombotic effects were observed 3 h after s.c. administration of Ixolaris and lasted for 24 h thereafter. *Ex vivo* experiments also showed that Ixolaris (up to 100 µg/kg) did not affect the aPTT, while the PT was increased by ~0.4-fold at the highest Ixolaris concentration. Remarkably, effective antithrombotic doses of Ixolaris (20 µg/Kg) was not associated with bleeding which was significant only at higher doses of the anticoagulant (40 µg/Kg). Our experiments demonstrate that Ixolaris is an effective and possibly safe antithrombotic agent *in vivo*.

Keywords

Anticoagulant; Ixolaris; venous thrombosis; factor Xa; tissue factor; tick saliva; *Ixodes scapularis*

Introduction

Following tissue injury, exposition of membrane-bound tissue factor (TF) is a crucial step in the initiation of blood coagulation. TF binds to blood coagulation factor VIIa (FVIIa), and the binary FVIIa/TF complex then generates FIXa and FXa (1–3). Generation of FIXa by the

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FVIIa/TF complex results in formation of the Xnase complex following binding to the nonenzymatic co-factor, activated factor VIII (FVIIIa). The Xnase complex, along with FVIIa/TF, converts FX to activated factor X (FXa), which assembles with activated factor V (FVa) into the prothrombinase complex that is directly responsible for the formation of thrombin (1–3).

Once generated, and depending on the particular vascular bed and rheologic environment, thrombin will convert fibrinogen to fibrin monomers, activate platelets, and eventually lead to thrombus development. Under pathologic conditions, clot formation will present clinically as deep vein thrombosis, pulmonary embolism, acute coronary syndrome, cerebrovascular events, peripheral arterial thrombosis, and even disseminated intravascular coagulation. Therefore, efforts to develop potent and specific antithrombotic agents are still a priority in cardiovascular medicine (4,5).

In addition to physiologic blood coagulation inhibitors tissue factor pathway inhibitor (TFPI), antithrombin, and heparin cofactor II (6,7), a number of specific inhibitors from exogenous sources have been identified from the salivary gland of blood-sucking arthropods (8,9). We have recently characterized Ixolaris, a two Kunitz-like TFPI from the tick *Ixodes scapularis* (10). Ixolaris binds to FXa or FX as scaffolds and inhibits extrinsic Xnase. Differently from TFPI, however, Ixolaris does not bind to the active site cleft of FXa. Instead, complex formation is mediated by the FXa heparin-binding exosite (11).

In the present study, we evaluated the *in vivo* effects of Ixolaris on thrombus formation using a thrombosis model in rats. After intravenous (i.v.) or subcutaneous (s.c.) administration, Ixolaris showed effective and long-lasting antithrombotic activity that was not associated with hemorrhage or bleeding. Therefore, Ixolaris may be used as a prototype to develop new anticoagulants targeting the extrinsic pathway.

Material and methods

Materials

Human FX was purchased from Haematologic Technologies (Essex Junction, VT). Activated partial thromboplastin time (aPTT) (cephalin plus kaolin) and prothrombin time (PT) (thromboplastin with calcium) reagents were from BioMérieux (Rio de Janeiro, Brazil). Anasedan (Xylazin) and Dopalen (Ketamin) were from Agribands (Rio de Janeiro, Brazil). NU-PAGE, MES buffer, and molecular weight markers were obtained from Invitrogen (Carlsbad, CA).

Animals

Adult Wistar rats (both sexes) weighing 200–250 g were housed under controlled conditions of temperature ($24 \pm 1^\circ\text{C}$) and light (12 h light starting at 07:00 h), and all experiments were conducted in accordance with standards of animal care defined by the Institutional Committee (Institute of Medical Biochemistry, Federal University of Rio de Janeiro).

Ixolaris expression and purification

Recombinant Ixolaris was expressed in High Five cells, using Baculovirus expression system (Invitrogen, San Diego, CA). Ixolaris was purified through affinity chromatography on a FX-Sepharose column (Pharmacia, Uppsala, Sweden) followed by a Macrosphere octadecylsilica column (Altech, Deerfield, IL). One single band was observed in 4% to 12% NU-PAGE gel with MES buffer.

Iodination of Ixolaris

^{125}I -labeled Ixolaris (^{125}I -Ixolaris) was prepared by iodination with [^{125}I] sodium iodide (CNEN, São Paulo, Brazil) in 200 $\mu\text{Ci}/\text{mg}$ of protein, using Iodogen (Sigma Chemical, St. Louis, MO) (100 $\mu\text{g}/\text{mg}$ of protein) following the manufacturer's instructions. Free iodide was removed by extensive dialysis against Tris-buffered saline, pH 7.4.

In vitro effect of Ixolaris on aPTT and PT

The *in vitro* effect of Ixolaris on coagulation tests aPTT and PT was evaluated on an Amelung KC4A coagulometer (Labcon, Heppenheim, Germany). Human blood samples were collected from healthy donors in 3.8% trisodium citrate (9:1, v/v), and platelet-poor plasma were obtained by further centrifugation at $2000\times g$ for 10 min. Rat plasma samples were obtained following the same procedure. Plasma (50 μl) was incubated with Ixolaris (10 μl) for 2 min at 37°C , followed by addition of aPTT reagent (50 μl , 1 min) and then 25 mM CaCl_2 (100 μl) or PT reagent (100 μl). Time for clot formation was then recorded.

Polyacrylamide gel electrophoresis

Electrophoresis was performed in polyacrylamide gels (PAGE) under non-denaturing conditions. ^{125}I -Ixolaris (4.0×10^4 cpm in 320 ng) and purified FX (4.5 μg) or human or rodent plasma were incubated at room temperature for 15 min and loaded onto a PAGE (10% acrylamide). Gels were further exposed to phosphorimaging (Storm, Amersham Biosciences, Uppsala, Sweden).

Stasis-induced thrombosis after injection of tissue thromboplastin

Thrombus formation by a combination of stasis and hypercoagulability was induced as described by Vogel et al. (12) with slight modifications (13). Wistar rats were anesthetized with xylazin (16 mg/kg, intramuscularly) followed by ketamin (100 mg/kg, intramuscularly). The abdomen was surgically opened, and after careful dissection the vena cava was exposed and dissected free from surrounding tissues. Two loose ligatures were prepared 1 cm apart on the inferior vena cava just below the left renal vein. Ixolaris at the indicated doses was administered i.v. (below the distal loose suture) or s.c. at different times before thrombosis induction. Tissue thromboplastin (3 mg/kg body weight) was injected into the vena cava, and stasis was immediately established by tightening the proximal suture. Tightening of the distal suture was performed 20 min after administration of thromboplastin, and the ligated segment was removed. The formed thrombus was removed from the segment, rinsed, blotted on filter paper, dried for 1 h at 60°C , and weighed. The protocol received official approval with regard to the care and use of laboratory animals.

Determination of radioactivity of ^{125}I -Ixolaris in rat blood

The *in vivo* distribution of ^{125}I -Ixolaris in rat blood was evaluated after s.c. administration. Samples containing $\sim 5.5 \times 10^6$ cpm (4 μg Ixolaris) were resuspended in 200 μl of phosphate-buffered saline (PBS) and administered by s.c. route. After 3, 20, 40, or 60 h, blood was collected by cardiac puncture in EDTA (5 mM, final concentration). Platelet-poor plasma was obtained by centrifugation, and aliquots of 500 μl were placed in glass test tubes. Radioactivity was determined in a gamma counter (LKB, Wallac, Finland).

Ex vivo effect of Ixolaris on aPTT and PT

The *ex vivo* effect of Ixolaris on aPTT and PT coagulation tests was evaluated on an Amelung KC4A coagulometer (Labcon). Ixolaris was administered s.c. to rats and after 24-h blood was collected by cardiac puncture in 3.8% trisodium citrate in (9:1, v/v). Platelet-poor plasma was obtained by centrifugation at $2000\times g$ for 10 min. Plasma (50 μl) was incubated for 1 min at

37°C followed by addition of aPTT reagent (50 µl, 1 min) and then 25 µM CaCl₂ (100 µl) or PT reagent (100 µl). Time for clot formation was then recorded.

Bleeding effect

A rat tail-transection model was used to evaluate the effect of Ixolaris on bleeding time. Ixolaris at appropriate doses was administered s.c. and animals were anesthetized after 3 or 24 h, as described above. The rat tail was cut 3 mm from the tip and carefully immersed in 40 ml of distilled water at room temperature. The hemoglobin content in water solution (absorbance at 540 nm) was used as an estimated of blood loss (14). Appropriate controls (s.c injection of PBS) were run in parallel.

Statistics

All data presented represent mean ± SD. Differences in mean values were analyzed using Student's t-test. P-values <0.05 were considered to be statistically significant.

Results

The *in vitro* anticoagulant effect of Ixolaris on human plasma is shown in Figure 1A. Ixolaris caused a dose-dependent increase in both PT and aPTT; however, Ixolaris showed a more pronounced effect toward the extrinsic pathway. This was not surprising since previous observations employing purified proteins showed that Ixolaris is potent inhibitor of the extrinsic Xnase complex (10). We further evaluated the anticoagulant properties of Ixolaris on rat plasma. Figure 1B shows a similar pattern of inhibition towards PT and aPTT. However, Ixolaris concentrations above 0.75 µg/ml seems to produce a more pronounced effect in both clotting tests with a more remarkable increase in the PT.

Ixolaris forms a complex with FX, as demonstrated by non-denaturing PAGE (10). In an attempt to detect Ixolaris binding to FX in plasma, we used ¹²⁵I-Ixolaris followed by identification of complex formation using non-denaturing PAGE. Figure 2, lane 1, shows the migration patterns obtained for ¹²⁵I-Ixolaris; and lane 2 shows ¹²⁵I-Ixolaris bound to purified FX, which displays a slower migration pattern. Figure 2 also shows that incubation of ¹²⁵I-Ixolaris with human (lane 3) or rat (lane 4) plasma produces a band with a similar migration pattern observed for ¹²⁵I-Ixolaris and purified FX. We have also tried to identify the Ixolaris-FX complex in plasma using monoclonal antibodies against the zymogen. This was not possible due to the highly heterogenous migration pattern exhibited by FX, under non-denaturing conditions, either in the absence or in the presence of Ixolaris.

To determine whether Ixolaris exerts antithrombotic action *in vivo*, we used a thrombosis model in rats that combines stasis and hypercoagulability (see Materials and methods). The control group that received tissue thromboplastin (3 mg/kg) showed 100% of thrombus formation, with a mean thrombus weight of 5.1 ± 0.26 mg (n = 4). In contrast, i.v. administration of Ixolaris produced a progressive decrease on thrombus formation, with a maximum effect observed at 10 µg/kg (Figure 3).

Next, the antithrombotic effect of Ixolaris was tested after s.c. administration. Figure 4A shows that Ixolaris was highly effective in preventing venous thrombosis by the s.c route, with complete inhibition attained with 20 µg/kg Ixolaris. We also evaluated the time dependence of antithrombotic activity of Ixolaris. Figure 4B shows that Ixolaris (20 µg/kg) completely abolished thrombus formation after 3 h of s.c. administration. The effect persisted for 24 h and was progressively reversed thereafter. To confirm the prolonged half-life of Ixolaris *in vivo*, a semiquantitative estimate of Ixolaris pharmacokinetics was obtained using ¹²⁵I-Ixolaris. Figure

5 shows that ^{125}I -Ixolaris concentration in plasma reached a peak after 3 h of s.c. administration and was 40% of the maximum value 20 h after administration of the anticoagulant.

The effects of Ixolaris on *ex vivo* clotting assays were then tested. Figure 6A shows that aPTT was not affected by 40 or 100 $\mu\text{g/kg}$ Ixolaris. In contrast, PT values were statistically higher in relation to control for Ixolaris doses higher than 20 $\mu\text{g/kg}$, showing a ~0.4-fold increase at 100 $\mu\text{g/kg}$.

Finally, the bleeding effect of Ixolaris was evaluated using a tail-transection model (Fig. 7); no significant bleeding was observed 3 or 24 h after Ixolaris administration (20 $\mu\text{g/kg}$). On the other hand, a statistically significant blood loss was observed 24 h after administration of 40 $\mu\text{g/kg}$ Ixolaris (see Discussion).

Discussion

Ixolaris is a two-Kunitz salivary gland protein that has been previously characterized as a potent inhibitor of the extrinsic Xnase (FVIIa-TF) complex (10). However, although Ixolaris presents extensive primary sequence homology with TFPI, it does not bind to the FXa catalytic site. On the contrary, complex formation is mediated by a number of surface-charged residues that constitute the FXa heparin-binding exosite (11). Another striking difference between Ixolaris and TFPI is the ability of the former to form a tight complex with zymogen FX. In fact, interaction of Ixolaris with FXa or FX is a prerequisite for inhibition of the extrinsic Xnase complex (10).

In the present study, we demonstrated that Ixolaris displays potent anticoagulant and antithrombotic activities. *In vitro* assays performed with human plasma demonstrated a robust prolongation of PT and, to a lesser extent, of aPTT. The prominent effect toward the extrinsic pathway (PT) clearly indicates the potent and preferential inhibitory activity toward the FVIIa-TF complex. The finding that Ixolaris at higher concentrations also affects the intrinsic pathway (aPTT) suggests that the inhibitor interferes with prothrombin conversion by FXa. In fact, we have recently demonstrated that Ixolaris is a specific ligand of the FXa heparin-binding exosite and impairs the productive assembly of the prothrombinase complex (11). These findings are corroborated by site-directed mutagenesis studies demonstrating that the FXa heparin-binding exosite contains a number of specific residues implicated in zymogen recognition (15,16).

As observed with purified FX, Ixolaris forms a stable, noncovalent complex with zymogen either in human or rat plasma. These observations lead us to test the inhibitor using an *in vivo* model of venous thrombosis. Ixolaris showed a potent antithrombotic activity following i.v. or s.c. administration in rats. Remarkably, Ixolaris displays very high potency ($\text{IC}_{50} = 7.5\text{--}15\text{ }\mu\text{g/kg}$) compared with that of other antithrombotic molecules tested in this same animal model. A number of sulfated polysaccharides including unfractionated heparin that potentialize either antithrombin or heparin cofactor II present antithrombotic activity in the range of 250–3000 $\mu\text{g/kg}$ (17,18). In addition, a number of direct thrombin inhibitors, including argatroban and hirudin variants, inhibit venous thrombosis with IC_{50} ranging from 170 to 250 $\mu\text{g/kg}$ and with half-lives below 60 min (19). It

Analysis of the time-dependence pattern of the antithrombotic action of Ixolaris (Figure 4) as well as the disappearance of ^{125}I -Ixolaris radioactivity in rat plasma (Figure 5) indicates a prolonged half-life. This is not surprising since Ixolaris is a tight ligand of FX which plasma half life is about 34–40 hours (20). Notably, no significant bleeding was observed 3h or 24 h after Ixolaris administration at concentrations that effectively blocked thrombus formation (20 $\mu\text{g/kg}$). In addition, blood loss was not observed 3 h after administration of Ixolaris at 40 $\mu\text{g/kg}$ but it was significant at 24 h. The slower distribution of Ixolaris after S.C. administration

at higher doses as compared to the distribution observed with 4 µg/kg ¹²⁵I-Ixolaris (Fig. 5) appears to accounts for the bleeding effects detected at later time points (Fig. 7).

This tight binding of Ixolaris to FX may represent an advantage when compared with other small molecular weight anticoagulants that display a shorter half life (5). A similar pharmacokinetic property has been observed in humans after administration of NAPc2, a hookworm anticoagulant that targets the extrinsic pathway and that has recently undergone clinical trials (21). It is important to recognize that administration of anticoagulants targeting FVIIa/TF such as Ixolaris, NAPc2, and TFPI appears to be effective *in vivo* and, notably, is most often not associated with major manifestations of bleeding (5,6,21,22).

In conclusion, Ixolaris displays potent antithrombotic effect *in vivo*, with prolonged half life and minor significant bleeding. Therefore, Ixolaris appears to be a promising anticoagulant for the prevention and treatment of venous or arterial thrombosis and/or in pathologic conditions with abnormal expression of TF (23–25).

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Abbreviations

aPTT	activated partial thromboplastin time
FX	factor X
FXa	factor Xa
FVIIa	factor VIIa
i.v	intravenous
PAGE	Electrophoresis was performed in polyacrylamide gel
PBS	phosphate-buffered saline
PT	prothrombin time
s.c	subcutaneous
TF	tissue factor
TFPI	tissue factor pathway inhibitor

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Figure 1. *In vitro* anticoagulant activity of Ixolaris

Ixolaris at the indicated doses was incubated with human (A) or rat (B) citrated plasma for 2 min. Coagulation aPTT (●) and PT (■) were determined as described in Materials and methods. Each point represents mean $\pm$ SD of three independent determinations.

Figure 2. Detection of FX-¹²⁵I-Ixolaris complexes by PAGE

Samples containing ¹²⁵I-Ixolaris and purified FX or human or rodent plasma were incubated at room temperature for 15 min and then loaded onto non-denaturing 10% PAGE. Gels were further exposed to phosphoimaging. Lane 1, 10 µl ¹²⁵I-Ixolaris (4.0×10^4 cpm); lane 2, 10 µl ¹²⁵I-Ixolaris (4.0×10^4 cpm) + 10 µl of human FX (10 µM); lane 3, 10 µl ¹²⁵I-Ixolaris (4.0×10^4 cpm) + 20 µl of human plasma; lane 4, 10 µl ¹²⁵I-Ixolaris (4.0×10^4 cpm) + 20 µl of rodent plasma. Arrows indicate bands corresponding to free and bound ¹²⁵I-Ixolaris.

Figure 3. Effect of Ixolaris on stasis-induced venous thrombosis in rats

Ixolaris at the indicated doses was administered i.v. 15 min before induction of thrombosis by thromboplastin (3 mg/kg) and stasis, as described in Materials and methods. Control group received PBS instead of Ixolaris. Each point represents mean $\pm$ SD of three to five animals. Asterisk, $P < 0.05$, as compared to values observed in the absence of Ixolaris.

Figure 4. Effect of Ixolaris on venous thrombosis in rats after s.c. administration

(A) Dose-dependence effect of Ixolaris on venous thrombosis following s.c. administration. The antithrombotic effects of Ixolaris, at the indicated doses, was evaluated after 3 h of drug administration. Thrombosis was induced as described in Material and methods. (B) Time-dependence effect of Ixolaris following s.c. administration. Ixolaris (20 $\mu\text{g/kg}$) was administered s.c. to animals, and thrombosis was induced after the indicated times. Control group (□) received PBS instead of Ixolaris. Each point represents mean $\pm$ SD of three to five animals. Asterisk, $P < 0.05$, as compared to values observed in the absence of Ixolaris.

Figure 5. Determination of blood distribution of ^{125}I -Ixolaris after s.c. administration

After the s.c. injection of ^{125}I -Ixolaris ($4\text{ }\mu\text{g}$; $5.5 \times 10^6\text{ cpm}$), blood samples were collected at the indicated times by cardiac puncture and platelet-poor plasma obtained by centrifugation. Aliquots of $500\text{ }\mu\text{l}$ of plasma were placed in glass test tubes, and radioactivity was determined in a gamma counter.

Figure 6. *Ex vivo* anticoagulant activity of Ixolaris

Ixolaris at the indicated concentrations was given s.c. to animals; after 24 h, blood was collected and platelet-poor plasma obtained. Coagulation tests aPTT (**A**) and PT (**B**) were determined as described in Materials and Methods. Each point represents mean $\pm$ SD of four or five animals. Asterisk, $P < 0.05$, as compared to values observed in the absence of Ixolaris.

Figure 7. Determination of the bleeding effect of Ixolaris

Ixolaris at the indicated doses was administered s.c.; after 3 or 24 h of administration, the rat tail was cut 3 mm from the tip. The tail was carefully immersed in 40 ml of distilled water at room temperature, and blood loss (hemoglobin content) was estimated at 540 nm after 60 min. The absorbance detected for a group that received PBS instead of Ixolaris was taken as control. Results represent the mean $\pm$ SD of five to ten animals. Asterisk, $P < 0.05$.