International Journal of Biological Macromolecules 78 (2015) 296–303

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Ecotin: Exploring a feasible antithrombotic profile

Luciana Serrão Wermelinger[a,b], Flávia Serra Frattani[a,b], Tatiana Correa Carneiro-Lobo[b], Charles S. Craik[c], Helena Carla Castro[d], Russolina Benedeta Zingali[b,*]

[a] Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia/CCS, Universidade Federal do Rio de Janeiro, Rio do Janeiro, RJ, Brazil
[b] Instituto de Bioquímica Médica Leopoldo de Meis/CCS, Universidade Federal do Rio de Janeiro, Rio do Janeiro, RJ, Brazil
[c] California Institute of Quantitative Biosciences (QB3), University of California, San Francisco, San Francisco, CA, USA
[d] Laboratório de Antibióticos, Bioquímica e Modelagem Molecular (LABioMol), Departamento de Biologia Celular e Molecular, IB-/CEG, Universidade Federal Fluminense, Niterói, RJ, Brazil

ARTICLE INFO

Article history:
Received 30 December 2014
Received in revised form 20 March 2015
Accepted 30 March 2015
Available online 13 April 2015

Keywords:
Ecotin
Thrombin inhibitor
Factor Xa inhibitor

ABSTRACT

Ecotin is an *Escherichia coli*-derived protein that can inhibit serine proteases. It has been suggested that this protein (ecotin-WT) and some of its variants could be used to develop a prototype to treat thrombosis. In this work, the effect of ecotin-WT and a variant of this protein harboring two mutations (Met84Arg and Met85Arg, ecotin-RR) were analyzed to determine their ability to prevent thrombus formation using *in vivo* models. Ecotins were analyzed *in vitro* using the coagulation tests. An *in vivo* venous thrombosis rat model and a pulmonary thromboembolism mouse model were used to investigate the antithrombotic activity. The bleeding time in rats using a tail-transection model was evaluated as a possible side effect caused by the administration of these proteins. Ecotin-RR was more effective in inhibiting thrombin than ecotin-WT. Both ecotins presented similar mechanisms of anticoagulation activity and were able to decrease thrombus formation. In contrast, only ecotin-RR increased survival rates in the *in vivo* pulmonary thromboembolism model, reinforcing the antithrombotic activity of ecotin-RR. Ecotin-WT and more so ecotin-RR showed potent antithrombotic effects, not associated with bleeding. The presented results indicate that ecotin-WT and ecotin-RR may be new scaffolds that could be used to develop anticoagulation molecules.

1. Introduction

The hemostatic system is essential to protect the integrity of the vasculature. Different processes are involved in this role, including the adhesion and activation of platelets and protein coagulation and inhibition factors [1]. The system is tightly regulated, and any minor imbalance may lead to serious pathological conditions, such as thrombosis [2]. Currently, venous thromboembolism (VTE) is a common pathology. Recent research has shown that the overall incidence of deep vein thrombosis (DVT) and pulmonary embolism (PE) involves 55 and 29 per 100,000 individuals each year, respectively [3,4]. In addition, the number of fatal cases observed 1 year after the first VTE event has an incidence rate of approximately 21%, demonstrating the importance of studying molecules to prevent and treat these conditions [5].

The pharmacologic prophylaxis and treatment of venous thrombosis are currently based on three types of anticoagulants: unfractionated heparin, low-molecular-weight heparins and vitamin K antagonists [6]. Their effects at multiple points in the coagulation cascade lead to severe bleeding problems and other side effects, such as thrombocytopenia, and require monitoring. Alternatively, direct thrombin inhibitor dabigatran and factor Xa inhibitors rivaroxaban and apixaban have been shown to be more selective and safer [7–9]. While these new drugs have fewer side effects, they are not ideal anticoagulants because they can cause bleeding and there are not specific antidotes [10,11]. The need for more efficient and safer anticoagulants is evident. Many efforts have been made, including extensive research using molecules from biological sources, such as hirudin (*Hirudo medicinalis*),

Abbreviations: Ecotin-WT, ecotin wild type; ecotin-RR, ecotin with two mutations at the primary binding site; TF.FVIIa, tissue factor–factor VIIa; PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time; *HEPES*, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); PEG-6000, polyethylene glycol 6000; S-2765, Z-D-Arg-Gly-Arg-p-nitroanilide; S-2238, *H-d*-Phe-Pip-Arg-*p*-nitroanilide; BAPNA, *N*α-benzoyl-DL-arginine 4-nitroanilide hydrochloride; PPP, platelet poor plasma; Tris–HCl, Trizma® hydrochloride; PBS, phosphate-buffered saline; u-PA, urokinase-type plasminogen activator.

* Corresponding author. Tel.: +55 21 3938 6782; fax: +55 21 3938 6782.
E-mail addresses: lwserrao@pharma.ufrj.br (L.S. Wermelinger), flaviafrattani@pharma.ufrj.br (F.S. Frattani), tatty.lobo@gmail.com (T.C. Carneiro-Lobo), charles.craik@ucsf.edu (C.S. Craik), hcastrorangel@vm.uff.br (H.C. Castro), lzingali@bioqmed.ufrj.br (R.B. Zingali).

http://dx.doi.org/10.1016/j.ijbiomac.2015.03.071
0141-8130/

ixolaris (*Ixodes scapularis*), bothrojaracin (*Bothrops jararaca* venom) and ecotin (*Escherichia coli*) [12–15].

Ecotin is a protein inhibitor of several serine proteases. It exists as a non-covalent homodimer formed by two 16,096 Da monomers in a head to tail association [16,17]. This homodimer is able to form a unique tetrameric enzyme–inhibitor complex with two serine proteases, such as FXa and trypsin [15,17]. Ecotin has two distinct sites that are known to interact with serine proteases: the primary binding site, which involves the reactive loop (80s loop, residues 81–86) and the 50s loop (residues 52–54), and the secondary binding site, which involves the surface loops 60s (residues 67–70) and 100s (residues 108–113) and the protease C-terminal region [18]. In addition, ecotin can inhibit serine proteases that are involved in several steps of the coagulation cascade, including FXa, FXIa, FXIIa and kallikrein [15,19,20]. However, no or little inhibition was observed with thrombin and the TF.FVIIa complex in the presence of ecotin wild type (ecotin-WT) [15]. Although thrombin was not inhibited by ecotin-WT, our group has shown that ecotin binds to human thrombin *via* its secondary binding site and modulates catalytic activity [21,22]. To improve its ability to inhibit several serine proteases, some mutations were performed in ecotins, including a single mutation of Met 84 to Arg, increasing ecotin's inhibition against thrombin, FXa, plasmin and FXIa and leading to a loss of its ability to inhibit elastase [15]. First, ecotin Met84Arg and Met85Arg was described as a potent inhibitor of urokinase-type plasminogen activator (uPa) [23]. Then, our group showed that ecotin with the mutations M84R and M85R changes the primary site of ecotin and it can then also interact with the active site of thrombin. An *in silico* study showed that P1 Arg^{84} electrostatically interacts with S1 (Asp^{189}) and P2 Arg^{85} forms a hydrogen bond with catalytic residues of thrombin, improving the affinity of this molecules and *in vitro* studies revealed the inhibitory capacity by preventing the platelet aggregation induced by thrombin [18,21,22,24,25].

While the anticoagulation effects of ecotin have been widely described in the literature, its actual antithrombotic effects were not demonstrated *in vivo* until now [15,20,22,26,27]. In this study, ecotin-WT and the mutant (Met84Arg and Met85Arg, ecotin-RR) were compared through a series of *in vitro* and *in vivo* coagulation, plasma clotting and thrombus formation models. The anticoagulation and antithrombotic properties of ecotin-WT and ecotin-RR were demonstrated and their potential uses in medicine are discussed.

2. Materials and methods

2.1. Animals

Male and female adult BALB-C mice (20–25 g) and Wistar rats (200–250 g) were maintained at room temperature under a 12 h light–dark cycle with unlimited access to food and water. The experiments followed the standards of animal care defined by the Center of Medical Sciences Committee at The Federal University of Rio de Janeiro (IBqM 004).

2.2. Proteins and chemicals

Ecotin-WT and ecotin-RR were expressed and purified as previously described [28]. Human thrombin was purified according to Ngai and Chang [29]. The following reagents were commercially obtained: human factor Xa (Calbiochem, San Diego, CA, USA), human fibrinogen and Z-D-Arg-Gly-Arg-p-nitroanilide (S-2765) and *H-d*-Phe-Pip-Arg-*p*-nitroanilide (S-2238) (Chromogenix, Stockholm, Sweden), bovine pancreatic trypsin, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid), HEPES, polyethylene glycol (PEG) 6000 and *N*α-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) (Sigma Chemical Co., St. Louis, USA). All of the solutions were freshly prepared.

2.3. Amidolytic activity of serine proteases

Hydrolysis of the synthetic substrates by human thrombin, human factor Xa and bovine trypsin were measured in 10 mM HEPES, 100 mM NaCl, 0.1% PEG 6000, pH 7.4, using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA) equipped with a microplate mixer and heating system. Human thrombin (2 nM), factor Xa (2 nM) or trypsin (2 nM) were added to the assay medium in the presence or absence of different concentrations of ecotins, and the reactions were started with the addition of S-2238, S-2756 chromogenic substrate (100 μM) or BAPNA (200 μM) with minor modifications by Mukherjee and Mackessy [30]. The initial rate of the *p*-nitroaniline release was determined by the increase in the Abs 405 nm in the first seconds. IC_{50} refers to the ecotin concentration that inhibited 50% of enzyme activity.

2.4. Fibrinogen clotting assay

Human fibrinogen clotting by human thrombin was measured using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA) [21]. Various ecotin concentrations were pre-incubated with thrombin (2 nM) for 5 min, and the reactions were started by the addition of 4 mg/mL human fibrinogen. IC_{50} refers to the ecotin concentration that inhibited 50% of clotting activity.

2.5. Anticoagulant activity assays: prothrombin time (PT) and activated partial thromboplastin time (APTT)

Wistar rats of 200–250 g were anesthetized with an intramuscular injection of ketamine (100 mg/kg body weight) and xylazine (16 mg/kg body weight). A BD Insyte™ Autoguard™ catheter (BD Medical) coupled to a 3 mL syringe with 3.8% trisodium citrate solution (1:9 citrate/blood, v/v) was inserted into the right carotid artery for blood collection. Platelet poor plasma (PPP) was obtained by centrifugation at 2000*g* for 20 min at room temperature. The plasma of Wistar rats (50 μL) was incubated for 1 min with different concentrations of ecotins for *in vitro* anticoagulation assays. For APTT tests, cephalin plus kaolin (APTT reagent, BioMeriaux, RJ, Brazil) were incubated for 2 min at 37 °C with pre-warmed plasma in the presence of different concentrations of both ecotins. The reaction was started by the addition of 100 μL of pre-warmed 25 mM $CaCl_2$. For PT tests, 100 μL of a pre-warmed solution of thromboplastin and calcium (PT reagent, BioMeriaux, RJ, Brazil) was added to 50 μL of pre-warmed plasma in the presence of different concentrations of both ecotins using an Amelung KC4A Coagulometer (Trinity Biotech, Germany) [31].

2.6. Venous thrombosis—stasis-induced thrombosis after injection of tissue thromboplastin in vivo

Thrombus formation through a combination of stasis and hypercoagulability was induced as previously described [32] with minor modifications by Mendes-Silva et al. [33]. Male and female Wistar rats of 200–250 g were anesthetized as described above. The abdomen was carefully opened and dissected, exposing the vena cava. The inferior vena cava just below the left renal vein was loosely tied at two points 1 cm apart from each other. Different concentrations of ecotins were administered intravenously below the distal tie, and the solution was allowed to circulate for 5, 30 or 60 min before thrombosis induction. The proximal tie was tightened, and stasis was induced through the injection of tissue thromboplastin (3 mg/kg body weight) into the vena cava. After 20 min of stasis, the thrombus in the occluded segment was