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Human cathelicidin LL-37 inhibits platelet aggregation and thrombosis via Src/PI3K/Akt signaling

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ABSTRACT

Biological functions of human cathelicidin LL-37 have been widely reported, including antibacterial, immune and anti-tumor effects. However, the antiplatelet activity of LL-37 has not been addressed. The purpose of our study was to investigate the antiplatelet and antithrombotic actions of LL-37. We found that this peptide inhibited human platelet aggregation *in vitro* and attenuated thrombus formation *in vivo*. Furthermore, LL-37 reduced phosphorylation of Src kinase and Akt^{Ser473}, decreased platelet spreading on immobilized fibrinogen and inhibited P-selectin expression on platelets. These results demonstrate that LL-37 has antiplatelet and antithrombotic actions.

1. Introduction

Platelet aggregation plays a key role in the process of normal hemostasis [1,2]. Once vascular injury occurs, the locally exposed Collagen/von Willebrand factor (vWF) complexes in the vascular wall bind to GPVI/GPIb-IX-V, producing a platelet monolayer that supports the subsequent adhesion of activated platelets to each other [3,4]. Additional platelets adhere to the initial monolayer and become activated [3]. Activated platelets release agonists from alpha and dense granules, such as adenosine diphosphate (ADP), thromboxane A_2 (TxA_2) and serotonin. These agonists contribute to amplifying signaling [1]. Once one or more agonists bind to their platelet membrane receptors, inside-out signaling leads to the conversion of αIIbβ3 (also known as glycoprotein IIb–IIIa or GPIIb/IIIa) from a low-affinity/avidity receptor to a high-affinity/avidity receptor. And αIIbβ3 can be occupied with soluble adhesive ligand, such as fibrinogen. As integrin ligation and clustering, outside-in signaling can be coordinated with other platelet membrane receptor signaling to regulate platelet activation, granule secretion, platelet spreading, clot retraction, platelet procoagulant activity and aggregation [5–7]. Among various signaling transmitter of platelet membrane receptors, the Src kinase, the phosphatidylinositol 3-kinase ($Akt^{Ser473/474}$ phosphorylation as the activation marker) and the mitogen activated protein kinases (p38 phosphorylation as the activation maker) are important.

Antimicrobial peptides (AMPs), from innate immune system of living organisms, are capable of eliminating a broad range of bacteria, viruses and fungi [8,9]. In mammals, two distinct groups of AMPs are defensins and cathelicidin [10,11]. hCAP-18 is the only human cathelicidin discovered in subpopulations of lymphocytes and monocytes, squamous epithelia, epididymis, seminal plasma, lung, and keratinocytes. It can be hydrolyzed by proteinase 3 between an alanyl and a leucyl residue to produce an antibacterial peptide LL-37 [12]. LL-37 was shown to kill bacteria, fungi and work as an antiviral agent [13]. In addition, LL-37 has been proposed to play a key role in chemotaxis, angiogenesis and wound healing [13,14]. However, the effect of LL-37 on platelet aggregation has not been addressed.

The aim of the current study was to investigate the antiplatelet and antithrombotic actions of LL-37, and its underlying mechanism.

2. Materials and methods

2.1. Antibodies and reagents

Peptide LL-37 was synthesized by Ningbo Kanbei Biochemistry (Ningbo, Zhejiang, China) with the purity of more than 98%. ADP,

Abbreviations: AMP, antimicrobial peptide; ADP, adenosine diphosphate; TxA_2, thromboxane A_2; GPIIb/IIIa, glycoprotein IIb–IIIa; MAPK, mitogen activated protein kinase; PI3K, phosphatidylinositol 3-kinase; SD, Sprague–Dawley; PRP, platelet-rich plasma; PPP, platelet-poor plasma; PVDF, poly vinylidene difluoride; PBS, phosphate buffer saline; BSA, albumin from bovine serum.

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thrombin, U46619 (TxA_2 analog), human fibrinogen, prostaglandin E1 (PGE1), FITC-labeled phalloidin and apyrase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Collagen was from Hyphen-Biomed (Neuvlle-sur-Oise, France). Fluorescently labeled monoclonal antibodies, anti-CD62P-PE, anti-CD42a-FITC, REA Control(S)-PE and REA Control(S)-FITC were obtained from Millipore (Temecula, CA, USA). Anti-Akt, anti-phospho-Akt, anti-p38, anti-phospho-p38, anti-Src, and anti-phospho-Src antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents used in this research were of analytical grade. cOmplete Protease Inhibitors and Phosphatase Inhibitors Cocktail Tablets were purchased from Roche Molecular Biochemicals (Indianapolis, IN, Switzerland). 4× loading buffer, 1% and 4% paraformaldehyde were purchased from Solarbio (Beijing, China).

2.2. Animals and human samples

Sprague–Dawley (SD) rats (180–220 g) were all purchased from Nanjing Qinglongshan Animal Center (Nanjing, Jiangsu, China). All animals were housed in a temperature-controlled environment under a reversed light and dark cycle, and fed with standard chow for at least one week before experiments. All experiments were carried out in accordance with the guidelines and the regulations of the Ethical Committee of China Pharmaceutical University. The protocols were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University. Human venous blood was obtained from healthy donors in accordance with the Declaration of Helsinki and the permission from the Ethical Committee of China Pharmaceutical University. The written informed consent was obtained from all participants.

2.3. Platelet preparation

Human venous blood was drawn from healthy donors without stasis into the siliconized vacutainers containing a 1:5 volume ACD (8.38 g of sodium citrate, 7.35 g of citric acid and 9.01 g of dextrose; pH 4.4). The blood samples were centrifuged at 142× g for 21 min to obtain PRP or at 278× g for 18 min to obtain PPP. Gel-filtered human platelets were prepared as previously described [15,16]. The gel-filtered human platelets collected from sepharose column were combined and adjusted to 2.5×10^8 platelets/ml using Tyrode's buffer (4.00 g of sodium chloride, 2.37 g of HEPES, 0.11 g of potassium chloride, 0.20 g of sodium dihydrogen phosphate, 0.50 g of BSA, 0.50 g of Glucose and 0.21 g of magnesium chloride in 500 ml of H_2O; pH 7.4). Washed human platelets were prepared as previously described with some modifications [17,18]. Briefly, PRP was centrifuged at 300× g for 10 min at room temperature in the presence of PGE1 (0.1 μg/ml), apyrase (1 U/ml) and EDTA (5 mM). After washing, platelets were re-suspended in Tyrode's buffer and adjusted to 3×10^7 platelets/ml.

2.4. Platelet aggregation assay

In vitro platelet aggregation was measured using the turbidimetric method with a four-channel aggregometer (LBY-NJ4, Pulisheng Instrument Co. Ltd., Beijing, China) as previously described [19,20]. Aliquots of gel-filtered human platelets at a concentration of 2.5×10^8 platelets/ml were preincubated with LL-37 or vehicle for 5 min at 37 °C. Then platelet aggregation was induced by ADP (10 μM), U46619 (3 μM), Collagen (2 μg/ml), or thrombin (0.26 U/ml). The maximum platelet aggregation rate was determined within 5 min with continuous stirring. The light transmittance was calibrated with PPP.

2.5. Arterio-venous shunt thrombosis in rats

Antithrombotic activity of LL-37 was tested in an arterio-venous shunt thrombosis model as described previously [21,22]. Fifty SD rats were randomly divided into 5 groups containing 10 rats in each group (males and females in half). The rats were treated with LL-37 (5, 10 and 15 mg/kg), aspirin (50 mg/kg) or vehicle. The weight of thrombus was determined 6 h later at room temperature by subtracting the weight of the dry 10-cm thread.

2.6. P-selectin expression

The expression of CD62P (P-selectin) was studied using a method described previously [15,23,24]. PRP were adjusted to 3×10^8 platelets/ml by adding Tyrode's buffer, and incubated with or without LL-37 (0.6 mM) for 10 min at 37 °C. The platelets were then stimulated with ADP (10 μM), Collagen (2 μg/ml) or U46619 (3 μM) in the presence of PE-labeled CD62P for 10 min at 2–8 °C and immediately fixed with 1% paraformaldehyde. Platelet-bound fluorescence was analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immuno-cytometry System, San Jose, CA, USA).

2.7. Immunoblot detection of Src, Akt^{Ser473} and p38 phosphorylation in platelets

Detection of Src, Akt^{Ser473} and p38 were performed as described previously [15,25]. Aliquots of gel-filtered platelets (2.5×10^8 platelets/ml) were preincubated with vehicle or LL-37 for 5 min at 37 °C and then stimulated by different agonist (ADP, U46619, Collagen or thrombin) for another 5 min under stirring at 37 °C. The reaction was stopped in RIPA buffer containing 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 10 mM Tris, 150 mM NaCl. Samples were heated for 5 min at 100 °C and stored at −20 °C. Proteins were analyzed by SDS-PAGE using 12% gels, and then transferred to a poly vinylidene difluoride (PVDF) membrane and immunoblotted via incubating with the corresponding antibodies. After incubation with the corresponding secondary antibodies at room temperature, densitometric band scanning was performed using a Bio-rad imaging systems.

2.8. Platelet spreading on immobilized fibrinogen

Analysis of platelets spreading on immobilized fibrinogen was conducted as previously described [26,27]. Glass coverslips were coated with fibrinogen (40 μg/ml) overnight, and then blocked with 2% BSA. Washed human platelets in Tyrode's buffer (3×10^7 platelets/ml) preincubated with LL-37 or vehicle for 5 min at 37 °C were allowed to spread on the fibrinogen-coated surfaces. After 1 h, non-adherent platelets were discarded, and surface-bound platelets were washed with phosphate buffer saline (PBS). Coverslips were fixed in 4% paraformaldehyde, and labeled with FITC-labeled phalloidin for 1 h in the dark. Then platelets were visualized by an upright fluorescent microscope AXIO ScopeA1 (ZEISS Group. Jena. Germany) equipped with a ×100 oil objective lens. Images were analyzed using National Institutes of Health Image J software (National Institutes of Health, Bethesda, MD).

2.9. Statistical analysis

The data are expressed as the mean of the replicate determinations and the standard deviation (SD) or standard error of measurement (SEM). Statistical significance was evaluated using student's t test for two-sample comparison or ANOVA followed by Dunnett's test for multiple comparisons. The data were analyzed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).