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A novel antimicrobial peptide isolated from centipede *Scolopendra subspinipes mutilans* stimulates neutrophil activity through formyl peptide receptor 2

Yoo Jung Park [a], Hyung Sik Kim [a], Ha Young Lee [a], Jae Sam Hwang [b], Yoe-Sik Bae [a, c, *]

[a] Department of Biological Sciences, Sungkyunkwan University, Suwon 16419, Republic of Korea
[b] Department of Agricultural Biology, National Academy of Agricultural Science, RDA, Wanju 55365, Republic of Korea
[c] Department of Health Sciences and Technology, SAIHST, Sungkyunkwan University, Seoul 06351, Republic of Korea

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ABSTRACT

In this study, we identified scolopendrasin X, a novel antimicrobial peptide (AMP), from centipede *Scolopendra subspinipes mutilans*. Scolopendrasin X strongly stimulated mouse neutrophils, resulting in intracellular calcium increase, chemotactic migration through pertussis toxin-sensitive G-protein and phospholipase C pathway, and increased superoxide anion production in neutrophils. Target receptor for scolopendrasin X, formyl peptide receptor (FPR)2 mediated scolopendrasin X-induced neutrophil activation. Moreover, scolopendrasin X significantly blocked inflammatory cytokine production induced by lipopolysaccharide in mouse neutrophils. Taken together, our results suggest that the novel AMP scolopendrasin X can be used as a material to regulate neutrophil activity through FPR2.

1. Introduction

The immune system can recognize invading pathogens and effectively remove them by innate immunity and adaptive immunity. In innate immune response, sentinel cells such as tissue residing macrophages can detect invading pathogen-associated molecular patterns using several pattern recognition receptors [1,2]. Activated macrophages then produce several inflammatory cytokines and chemokines, leading to recruitment of leukocytes from peripheral blood vessel into infected area [3]. Neutrophils are the first leukocytes recruited into the event area after sensing cytokines and chemokines [4]. Neutrophils can also detect pathogen-derived molecules such as N-formyl peptides to mediate innate immune response [4,5]. Many extracellular stimuli can regulate neutrophil activity in innate immune response. Identifying molecules that can stimulate neutrophils would be important to control innate immunity.

Centipede *Scolopendra subspinipes mutilans* has been used in oriental medicine to treat several human diseases, including rheumatoid arthritis, lymphadenopathy, and carcinoma [6,7]. This centipede also possesses antimicrobial ingredients that can protect lung and intestine against infection [8]. However, molecular identities of bioactive components in this centipede that can modulate disease pathogenesis have not been extensively revealed due to limited information about its genome analysis. In this study, we systematically analyzed the genome of *Scolopendra subspinipes mutilans* to identify novel antimicrobial peptides (AMPs) according to a previous report [9]. We identified scolopendrasin X (sequence: MKKFHCLKKICKGLCAKL-$CONH_2$), a novel AMP from *Scolopendra subspinipes mutilans.* It strongly stimulated mouse neutrophils, resulting in calcium increase, chemotactic migration, and superoxide anion production. We also found that formyl peptide receptor (FPR)2, an important classical chemoattractant receptor, could mediate scolopendrasin X-induced neutrophil activation.

2. Materials and methods

2.1. Materials

Scolopendrasin X, WKYMVm, MMK-1, and WRW4 were synthesized by Anygen (Gwangju, Korea) with purity > 99.6%. fMLF was purchased from Sigma-Aldrich (St. Louis, MO, USA). Boyden

* Corresponding author. Department of Biological Sciences, Sungkyunkwan University, Suwon 16419, Republic of Korea.
E-mail address: yoesik@skku.edu (Y.-S. Bae).

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chambers were purchased from Neuroprobe, Inc. (Gaithersburg, MD, USA). Fura-2 pentaacetoxymethylester (fura-2/AM) was purchased from Molecular Probes (Eugene, OR, USA). Pertussis toxin (PTX) was purchased from Calbiochem (San Diego, CA, USA). 1-[6-((17β-3-methoxyestera-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione (U-73122) and 1-[6-((17β-3-methoxyestera-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione (U-73343) were obtained from Calbiochem (San Diego, CA, USA). RPMI 1640 was obtained from Welgene (Gyeongsan, Korea).

2.2. Isolation of mouse neutrophils

Mouse bone marrow neutrophils were isolated according to a previous report [10]. Briefly, mouse bone marrow cells were isolated from femurs and tibias of C57/BL6 mice and suspended in HBSS-EDTA solution. Isolated mouse bone marrow cells were centrifuged at 400*g* for 10 min. Resuspended cells in HBSS-EDTA solution were then carefully loaded onto a 52%/69%/78% Percoll gradient and centrifuged at 1500*g* for 30 min. After isolating cells in the 69%/78% interface layer, red blood cells were removed by hypotonic lysis. Isolated cells were then stained with anti-Ly6G antibody and found to be over 95% Ly6G-positive. They were then subjected to flow cytometry (BD FACSCanto II).

2.3. Intracellular calcium measurement

Intracellular calcium concentration was measured using fura-2/AM according to a previous report [11]. Briefly, isolated mouse neutrophils, vector-, FPR1-, or FPR2-expressing RBL-2H3 cells were loaded with 3 μM fura-2/AM at 37 °C for 50 min in fresh serum-free RPMI 1640 medium. Fura-2/AM loaded cells (1×10^7) in Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES, pH 7.3, 10 mM glucose, 2.2 mM $CaCl_2$, and 0.2 mM EGTA) were aliquoted for each assay. Fluorescence changes at dual excitation wavelengths of 340 nm and 380 nm with emission wavelength of 500 nm were measured after stimulating cells with each stimulus. To test the effect of U-73122 or U-73343 on scolopendrasin X-induced calcium increase, 5 μM of each compound was used for pre-treatment for 3 min before scolopendrasin X stimulation.

2.4. Superoxide anion measurement

Superoxide anion production was measured based on cytochrome *c* reduction assay using a microtiter 96-well plate ELISA reader (Bio-Tek instruments, EL312e, Winooski, VT, USA) as described previously [12]. Briefly, isolated mouse neutrophils (1×10^6 cells/100 μl of RPMI 1640 medium per well of a 96-well plate) were stimulated with different stimuli in the presence of 50 μM cytochrome *c* and 5 μM cytochalasin B. Superoxide generation was measured as a change in light absorption at 550 nm over 10 min at interval of 1 min.

2.5. Chemotaxis assay

Chemotaxis assays with mouse bone marrow neutrophils and vector-, FPR1-, or FPR2-expressing RBL-2H3 cells were performed

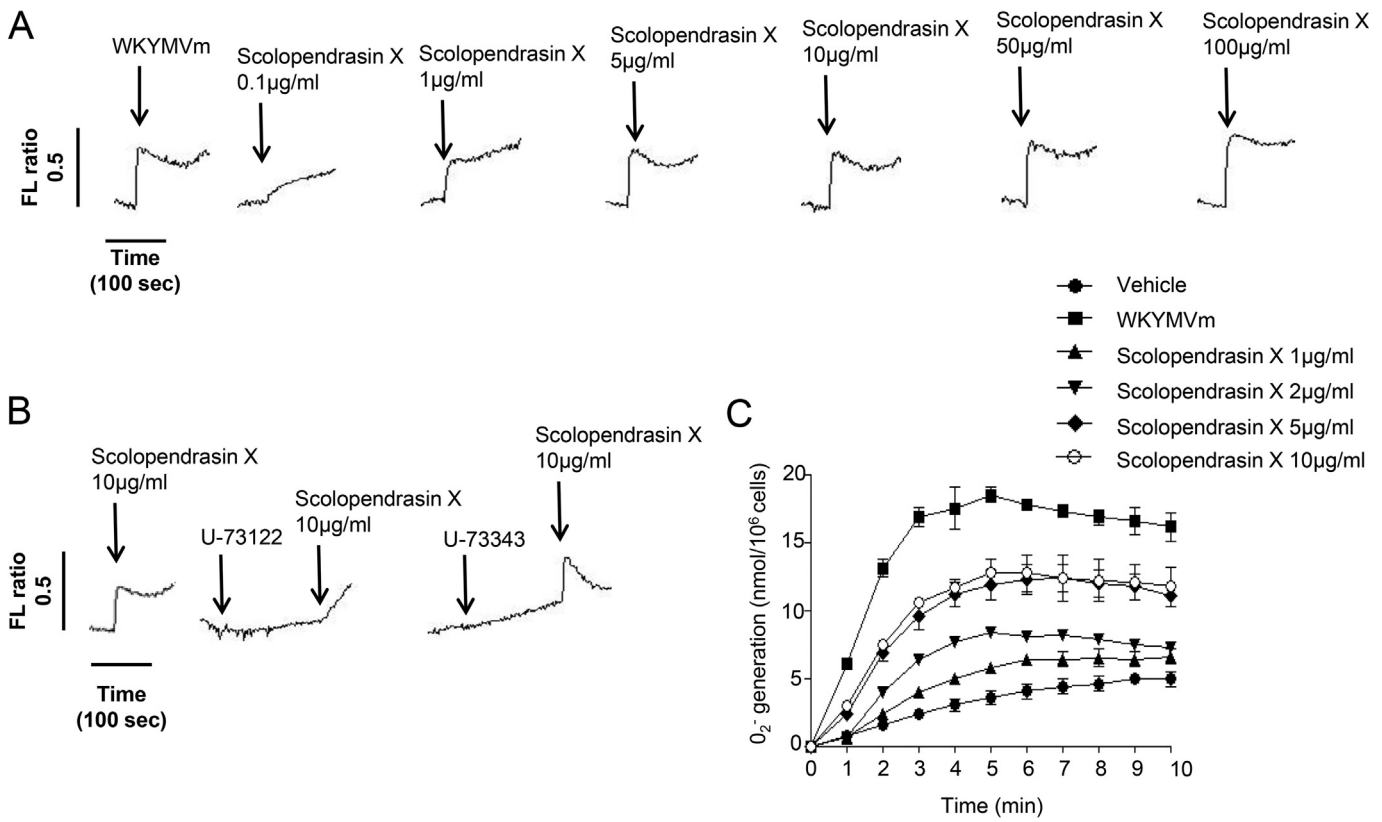

Fig. 1. A novel AMP scolopendrasin X stimulates neutrophils, resulting in calcium increase and superoxide anion production. (A) Mouse neutrophils were treated with several concentrations (0.1 μg/ml, 1 μg/ml, 5 μg/ml, 10 μg/ml, 50 μg/ml, and 100 μg/ml) of scolopendrasin X or WKYMVm (1 μM). (B) Mouse neutrophils were incubated without or with 5 μM U-73122 or 5 μM U-73343 for 3 min and stimulated with 10 μg/ml of scolopendrasin X. Relative intracellular Ca^{2+} concentrations are expressed as fluorescence ratios (340:380 nm) (A, B). Data are representatives of three independent experiments (A, B). (C) Mouse neutrophils (1×10^6 cells/100 μl of RPMI 1640 medium per well of a 96-well plate) were stimulated with different concentrations (0 μg/ml, 1 μg/ml, 2 μg/ml, 5 μg/ml, and 10 μg/ml) of scolopendrasin X or 1 μM of WKYMVm. Superoxide anion production was determined by measuring cytochrome *c* reduction (C). Data are presented as means ± standard error (n = 3).

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