Promotion of formyl peptide receptor 1-mediated neutrophil chemotactic migration by antimicrobial peptides isolated from the centipede Scolopendra subspinipes mutilans

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Supplementary Materials and Methods

Materials

Scolopendrasin III, scolopendrasin V, WKYMVm, and MMK-1 were synthesized by Anygen (Gwangju, Korea) with a purity > 99.6 %. fMLF and CsH were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Boyden chambers were purchased from Neuroprobe, Inc. (Gaithersburg, MD, USA). Antibodies against phosphorylated kinases including anti-phospho-Akt, anti-phospho-ERK were purchased from Cell Signaling Technology (Beverly, MA, USA). PD98059 was purchased from Calbiochem (San Diego, CA, USA). LY294002 was from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, USA). MK-2206 and p-nitrophenyl-N-acetyl-β-D-glucosamidine were purchased from Sigma (St. Louis, MO,
USA). Fura-2 pentaacetoxymethylester (fura-2/AM) was purchased from Molecular Probes (Eugene, OR, USA). RPMI 1640 was obtained from Welgene (Gyeongsan, Korea). FITC-conjugated anti-human FPR1 mouse IgG was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Anti-Ly6G and anti-CD11b antibodies were obtained from e-Bioscience. Inc. (San Diego, CA, USA).

Isolation of mouse neutrophils
Mouse bone marrow neutrophils were isolated as previously described (30). Briefly, bone marrow cells isolated from femurs and tibias were suspended in HBSS-EDTA solution. After centrifugation at 400g for 10 min, resuspended cells were carefully loaded on a 52%/69%/78% Percoll gradient and centrifuged at 1500g for 30 min. Cells were isolated in the 69%/78% interface layer, then RBCs were removed by hypotonic lysis. Isolated cells were over 95% Ly6G-positive as assessed by flow cytometry (BD FACSCanto II).

Chemotaxis assay
Chemotaxis assays with mouse bone marrow neutrophils and vector-, FPR1-, or FPR2-expressing RBL-2H3 cells were performed in accordance with a previous report using a multiwell Boyden chamber (31). Cells were applied to different-sized polycarbonate filters (3 μm pore size for mouse neutrophils, 5 μm pore size for mouse bone marrow-derived macrophages, and 8 μm pore size for the RBL-2H3 cells) for 90 min (2 h for the macrophages, 4 h for the RBL-2H3 cells) at 37°C. After removing non-migrated cells, the migrated cells were stained with hematoxylin (Sigma, St. Louis, MO, USA) and then quantified using a light microscope as previously described (31).

Measurement of superoxide anion production
Superoxide anion generation was determined by measuring reduction of cytochrome c using a microtiter 96 well plate ELISA reader (Bio-Tek instruments, EL312e, Winooski, VT, USA) as described (31). Isolated mouse neutrophils ($1 \times 10^6$ cells/100µl of RPMI 1640 medium per well of a 96-well plate) were stimulated with peptides in the presence of 50 µM cytochrome c and 5 µM cytochalasin B. The superoxide generation was measured as change in light absorption at 550 nm over 5 minutes at 1 min intervals.

**Measurement of degranulation activity**

Degranulation activity was determined by measuring the amount of released β-hexosaminidase as described previously (21). Isolated mouse neutrophils ($1 \times 10^6$/well) were washed twice with Tyrode’s buffer (137 mM NaCl, 12 mM NaHCO₃, 5.6 mM glucose, 2.7 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.4 mM NaH₂PO₄, 0.1% BSA, and 25 mM HEPES, pH 7.4) and stimulated with individual peptides. The reaction was terminated 30 min after stimulation by placing the plate on ice. The amount of β-hexosaminidase secreted into the medium was determined by incubating supernatant or cell lysate using 5 mM p-nitrophenyl-N-acetyl-β-D-glucosamide in 0.1 M sodium citrate buffer (pH 3.8).

**Measurement of intracellular calcium concentration**

The level of intracellular calcium concentration was measured by Grynkiewicz's method using fura-2/AM (32). Isolated mouse neutrophils were incubated with 3 µM fura-2/AM at 37°C for 50 min in fresh serum free RPMI 1640 medium under continuous stirring. $1 \times 10^7$ cells were aliquoted for each assay in Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl₂, 5 mM HEPES, pH 7.3, 10 mM glucose, 2.2 mM CaCl₂, and 0.2 mM EGTA). The fluorescence changes at the dual excitation wavelengths of 340 nm and 380 nm and the
emission wavelength of 500 nm were measured.

**Measurement of surface expression of FPR1**

Vector- or FPR1-expressing RBL-2H3 cells were incubated with FITC-conjugated monoclonal antibodies against FPR1 or isotype control for 40 min, followed by washing with FACS buffer (PBS containing 5% BSA). The samples were analyzed using a flow cytometer (FACScanto II, BD).

**Western blot analysis**

Isolated mouse neutrophils were stimulated with 100 μg/ml of Scolopendrasin III or Scolopendrasin V for 0 min, 2 min, 5 min, 10 min, and 30 min. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with specific antibodies (anti-phospho-ERK, anti-phospho-Akt, or anti-β-actin), and antigen–antibody complexes were visualized after the membrane was incubated with goat anti-rabbit IgG antibodies combined with horseradish peroxidase. Protein levels were detected using an enhanced chemiluminescence assay as previously described (33).

**Generation of bone marrow-derived macrophages**

Mouse bone marrow-derived macrophages were generated as previously described (10). Briefly, the bone marrow cells were isolated by flushing the femurs and tibias of wild-type C57BL/6 mice with ice-cold PBS. Bone marrow progenitor cells were cultured in 10 % FBS containing α-MEM under standard incubator conditions for 1 day. After removing the adherent cells, 10 % FBS containing α-MEM with 30 ng/ml M-CSF was added, and the cells were cultured for 3 days.
**In vivo neutrophil migration assay**

C57/BL6 mice were purchased from Orient Bio Inc. (Seongnam, Korea). FPR1-deficient mice were kindly provided by Dr. P. Murphy (NIAID, National Institutes of Health, Bethesda, MD, USA) (34). Vehicle or the two AMPs (15mg/kg) were administrated into the peritoneal cavity of WT C57/BL6 mice or FPR1-deficient mice for 2 h. Peritoneal fluid was collected, and then cells were stained with anti-Ly6G or anti-CD11b antibody. Neutrophils that were recruited into the peritoneal fluid were analyzed by flow cytometry.

**Data analysis**

Results are expressed as mean ± S.E. The Student’s *t*-test was used to compare individual treatments with their respective control values. Statistical significance was set at *p* < 0.05.

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