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

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We investigated the effects of two antimicrobial peptides (AMPs) isolated from *Scolopendra subspinipes mutilans* on neutrophil activity. Stimulation of mouse neutrophils with the two AMPs elicited chemotactic migration of the cells in a pertussis toxin-sensitive manner. The two AMPs also stimulated activation of ERK and Akt, which contribute to chemotactic migration of neutrophils. We found that AMP-stimulated neutrophil chemotaxis was blocked by a formyl peptide receptor (FPR) 1 antagonist (cyclosporin H); moreover the two AMPs stimulated the chemotactic migration of FPR1-expressing RBL-2H3 cells but not of vector-expressing RBL-2H3 cells. We also found that the two AMPs stimulate neutrophil migration in vivo, and that this effect is blocked in FPR1-deficient mice. Taken together, our results suggest that the two AMPs stimulate neutrophil migration through FPR1, and the two AMPs will be useful for the study of FPR1 signaling and neutrophil activation. [BMB Reports 2016; 49(9): 520-525]

### INTRODUCTION

Neutrophil migration is an important event in the immune response against infection or tissue damage (1). Tissue-resident sentinel cells such as macrophages and dendritic cells recognize and capture invading pathogens, resulting in the activation of the sentinel cells and the production of chemokines (2). The chemokines cause recruitment of circulating neutrophils to the blood to the infected site (3, 4). In addition to chemokines, invading bacteria-derived molecules also cause the recruitment of neutrophils into infected sites (3, 4). One important bacteria-derived neutrophil recruiting molecule is formyl peptide (5). As chemotactic neutrophil migration into sites of infection or injury is crucial in regulating immune or inflammatory responses, it is important to identify new molecules that can control neutrophil migration.

*Scolopendra subspinipes mutilans* has been used in oriental medicine to treat rheumatoid arthritis, lymphadenopathy, and carcinoma (6, 7). However, the identities of the bioactive components that regulate the activity of leukocytes or other cell types have not been extensively studied. In a previous report, we identified some antimicrobial peptide (AMP) candidates through de novo RNA sequencing from *Scolopendra subspinipes mutilans* (8). We demonstrated that some of the AMPs have dual activity, with bactericidal activity and anti-cancer activity against leukemia (9). Recently we also reported that one AMP isolated from *Scolopendra subspinipes mutilans* stimulates macrophage chemotaxis (10). In this study, we asked whether AMPs isolated from *Scolopendra subspinipes mutilans* stimulate neutrophil activity. We found that two AMPs from *Scolopendra subspinipes mutilans* [scolopendrasin III (sequence: VIYVHCTSSRGNNVRLLCAKSKVAP-NH₂), scolopendrasin V (sequence: YYGGGYKYKHWGCR-NH₂)] strongly stimulate neutrophil chemotaxis. We also revealed that neutrophil chemotaxis induced by the two AMPs is mediated by an important neutrophil chemoattractant receptor, formyl peptide receptor (FPR) 1.

### RESULTS

Two novel AMPs stimulate neutrophil chemotactic migration in a pertussis toxin (PTX)-sensitive manner

Previously, we and others demonstrated that AMPs, including LL-37 and scolopendrasin VII, stimulate chemotactic migration...
of leukocytes (10, 11). In this study, we tested the effects of two new AMPs (scolopendrasin III and scolopendrasin V) isolated from *Scolopendra subspinipes mutilans* on neutrophil activity. Because the three scolopendrasin AMPs have antimicrobial activity, we compared the amino acid sequence between the three scolopendrasin AMPs using multiple sequence alignment with hierarchical clustering as described previously (12). We found that some amino acids are commonly conserved between two of the AMPs among three scolopendrasin AMPs (Fig. 1A). However, well-conserved amino acids were not detected among the three AMPs (Fig. 1A). We examined the effects of the two new AMPs on neutrophil chemotaxis using a Boyden chamber assay kit. Stimulation of mouse neutrophils with the two AMPs strongly elicited neutrophil chemotactic migration (Fig. 1B). AMPs-induced neutrophil chemotactic migration was concentration-dependent, and maximal activity was induced at 100 μg/ml AMP (Fig. 1B).

Many chemokines and chemoattractants induce neutrophil chemotaxis via PTX-sensitive G-protein(s) (13, 14). Therefore, we also investigated whether AMP-induced neutrophil chemotaxis is mediated by PTX-sensitive G-protein(s). Preincubation of neutrophils with PTX prior to the chemotaxis assay with the two AMPs almost completely abolished peptide-induced neutrophil chemotaxis (Fig. 1C). In a positive control experiment, we found that WKYMVm-induced neutrophil chemotaxis was also completely blocked by PTX treatment (Fig. 1C). The results indicate that the two AMPs stimulate neutrophil chemotactic migration via PTX-sensitive G-protein(s).

In addition to chemotactic migration into the infectious or injured area, neutrophils mediate immune responses by stimulating production of superoxide anion (15). We examined whether the two AMPs stimulate superoxide anion production from mouse neutrophils using cytochrome c reduction assay. As shown in Fig. 1D, both AMPs failed to stimulate superoxide anion production. However, the well-known FPR agonist WKYMVm strongly stimulated the production of superoxide anion from neutrophils (Fig. 1D). Degranulation is also important to mediate immune responses against infection (16). The two new AMPs did not stimulate degranulation in neutrophils (Fig. 1E).

**Two new AMPs selectively stimulate intracellular signaling downstream of PTX-sensitive G-protein(s)**

There are wide range of extracellular stimuli that elicit diverse intracellular signaling cascades, which mediate various cellular responses. Intracellular calcium increase is an important event, which is associated with neutrophil activation in response to extracellular stimuli (17). In this study, we examined the effects of the two AMPs on calcium signaling in mouse neutrophils. Neither of the AMPs stimulated intracellular calcium increase in neutrophils (Fig. 2A). However, stimulation of mouse neutrophils with WKYMVm strongly elicited intracellular calcium increase (Fig. 2A). ERK is known to play crucial roles in the regulation of neutrophil activity (18). We tested the effects of the two AMPs on the activity of the ERK by monitoring phosphorylation of the enzymes. Stimulation of mouse neutrophils with the two AMPs...
Fig. 2. The two AMPs stimulate ERK and Akt activity in mouse neutrophils. (A) Fura-2 loaded mouse neutrophils were stimulated with scolopendrasin III (100 µg/ml), scolopendrasin V (100 µg/ml), or 1 µM WKYMVm, and intracellular calcium levels were determined fluorometrically using spectrophotometer. The peak levels of intracellular calcium were recorded. (B) Mouse neutrophils were stimulated with 100 µg/ml of Scolopendrasin III (left) or Scolopendrasin V (right) for several lengths of time (0 min, 2 min, 5 min, 10 min, 30 min). Total cell lysates were separated SDS-PAGE, and the levels of p-ERK and p-Akt were measured using Western blot analysis. Data are representative of three independent experiments (A, B). (C) Mouse neutrophils were incubated in the absence or presence of PD98059 (50 µM) for 60 min, LY294002 (50 µM) for 15 min, or MK-2206 (2 µM) for 20 min, and were applied to the upper well of a multwell chamber containing 100 µg/ml of Scolopendrasin III (left) or Scolopendrasin V (right) for 90 min. The number of migrated cells was determined by counting under a light microscope. Data are presented as means ± S.E. (n = 2). Data in the panels are representative of three independent experiments performed in duplicate (C).

markedly induced phosphorylation of ERK (Fig. 2B). Phosphorylation of ERK was transiently induced by the two AMPs, showing time-dependent manner. Scolopendrasin III-induced ERK phosphorylation was transient showing maximal activity at 2-5 min and returning to basal levels at 10 min (Fig. 2B left). Scolopendrasin V-induced ERK phosphorylation was apparent at 5-30 min after stimulation (Fig. 2B right). Because the two AMPs stimulated chemotactic migration of neutrophils, we tested the roles of ERK on AMP-induced neutrophil chemotaxis using specific inhibitor of ERK (PD98059). Pre-incubation of neutrophils with an ERK inhibitor (PD98059) prior to the chemotaxis assay almost completely inhibited peptide-induced chemotaxis (Fig. 2C). These results indicate that neutrophil chemotaxis induced by the two AMPs is mediated by the activity of ERK.

Akt has also been reported to play a key role in the regulation of cell migration (19). In our study, we tested whether the two AMPs stimulate Akt activity. Stimulation of mouse neutrophils with the two AMPs elicited Akt phosphorylation at 2 to 10 min after stimulation (Fig. 2B). Pretreatment of neutrophils with Akt inhibitor (MK-2206) or phosphatidylinositol 3-kinase (PI3K) (upstream molecule of Akt) inhibitor (LY294002) prior to the chemotaxis assay almost completely inhibited peptide-induced neutrophil chemotaxis, indicating that AMP-induced chemotaxis is mediated by PI3K/Akt pathway (Fig. 2C).

FPR1 mediates neutrophil chemotaxis induced by the two AMPs

Our finding that the two AMPs stimulate neutrophil chemotaxis, which is completely inhibited by PTX, led us to test the possible role of a well-known neutrophil chemoattractant receptor, FPR1, which is a PTX-sensitive G protein-coupled receptor. At first we examined the effect of an FPR1 antagonist, cyclosporin H (CsH) (20), on neutrophil chemotaxis stimulated by the two AMPs. Neutrophil chemotaxis was almost completely blocked by CsH. CsH also completely inhibited fMLF-induced neutrophil chemotaxis (Fig. 3A). To confirm that the AMP-induced neutrophil chemotaxis is mediated by FPR1, we used stably transfected FPR1-expressing RBL-2H3 cells (21). First, we used flow cytometric analysis to confirm that FPR1-expressing RBL-2H3 cells, but not the vector-expressing RBL-2H3 cells, express FPR1 on the cell surface (Fig. 3B). Next, we tested the effect of the two AMPs on chemotactic migration in vector- or FPR1-expressing RBL-2H3 cells. The two AMPs markedly stimulated chemotactic migration of cells in FPR1-expressing but not vector-expressing RBL-2H3 cells (Fig. 3C). The well-known FPR1 agonist fMLF also strongly induced the chemotactic migration of FPR1-, but not of the vector-expressing RBL-2H3 cells (Fig. 3D). However, neither of the AMPs stimulated the chemotactic migration of cells in the FPR2-expressing RBL-2H3 cells (Fig. 3D). These results suggest that FPR1 but not FPR2 mediates the neutrophil chemotaxis induced by the two AMPs. Since the two new AMPs stimulate neutrophil chemotaxis via FPR1, we also investigated whether the two AMPs induce the chemotactic migration of macrophages, which also express FPR1. As expected, the two AMPs significantly stimulated the chemotactic migration of macrophages (Fig. 3E).
The two AMPs elicit neutrophil recruitment in vivo via FPR1

Since we observed that the two AMPs stimulate neutrophil chemotaxis in vitro, we then tested their effect on recruitment of neutrophils in vivo. Vehicle administration did not induce neutrophil recruitment into the peritoneal cavity, and no significant difference was observed in either WT or FPR1-deficient mice (Fig. 4A). Administration of the two AMPs into the peritoneal cavity strongly induced recruitment of neutrophils into the peritoneal cavity (Fig. 4B, 4C). Because we found that the two AMPs stimulate neutrophil chemotaxis through FPR1 at the cellular level, we also examined the functional role of FPR1 on AMP-induced in vivo neutrophil recruitment using FPR1-deficient mice. Neutrophil recruitment into the peritoneal cavity induced by the two AMPs was
intracellular calcium increase, mediating diverse cellular responses (25, 26). We investigated whether the two AMPs stimulate intracellular calcium increase using Fura-2/AM, a calcium binding dye. Stimulation of fura-2/AM-loaded neutrophils with the two AMPs did not increase intracellular calcium levels (Fig. 2A). However, a well-known neutrophil stimulant, WKYMVm, strongly induced intracellular calcium increase in the cells (Fig. 2A). Taken together, our results show that the two novel FPR1 agonists stimulate PTX-sensitive G protein/ERK/Akt pathways, leading to chemotactic migration of neutrophils, without affecting intracellular calcium release and subsequent degranulation and superoxide anion production from neutrophils. In a previous report, we demonstrated that FPR1 is differentially activated by different agonists in a ligand-selective manner (21). Unlike WKYMVm, the WKGMVm and WKRMVm stimulated phosphorylation of ERK/Akt and the subsequent chemotactic migration, but did not stimulate calcium increase and subsequent degranulation activity in FPR1-expressing RBL-2H3 cells (21). Considering that superoxide anion production and degranulation are mediated by intracellular calcium increase in neutrophils (27, 28), our finding suggests that the activation of FPR1 by the two new AMPs do not induce calcium increase-mediated superoxide anion production and degranulation in the cells. The two new AMPs stimulate FPR1, showing selective activation of FPR1 downstream signaling leading to ERK/Akt phosphorylation and subsequent chemotactic migration. Previously, it has been suggested that one G-protein coupled receptor can be activated by different ligands via ligand-selective receptor activation states (29). Binding of different ligands to one G-protein coupled receptor may induce distinct receptor conformation change, resulting in coupling of the receptor to different heterotrimeric G-protein(s), and subsequent activation of different effector enzymes (29). Studies on the detail mechanism involved in the differential activation of FPR1 by the two new AMPs or WKYMVm are needed. In conclusion, in this study we report that the two AMPs (scolopendrasin III and scolopendrasin V) are novel FPR1 agonists. The two AMPs can be used as biased agonists for the selective activation of FPR1 downstream signaling.

MATERIALS AND METHODS

Materials and Methods are available in the Supplementary Data.

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REFERENCES

1. Kolaczkowska E1 and Kubes P (2013) Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol 13, 159-175
2. Heit B, Robbins SM, Downey CM et al (2008) PTEN functions to 'prioritize' chemotactic cues and prevent 'distraction' in migrating neutrophils. Nat Immunol 9, 743-752
3. Wright HL, Moots RJ, Bucknall RC et al (2010) Neutrophil function in inflammation and inflammatory diseases. Rheumatology 49, 1618-1631
4. Craig A, Mai J, Cai S and Jeyaseelan S (2009) Neutrophil recruitment to the lungs during bacterial pneumonia. Infect Immun 77, 568-575
5. Fu H, Karlsson J, Bylund J et al (2006) Ligand recognition and activation of formyl peptide receptors in neutrophils. J Leukoc Biol 79, 247-256
6. Yang M, Xiao C, Wu Q et al (2010) Anti-inflammatory effect of Sanshuibaie decoction may be associated with nuclear factor-kappa B and p38 MAPK alpha in collagen-induced arthritis in rat. J Ethnopharmacol 127, 264-273
7. Zhao H, Li Y, Wang Y et al (2012) Antitumor and immunostimulatory activity of a polysaccharide-protein complex from Scolopendra subspinipes mutilans L Koch in tumor-bearing mice. Food Chem Toxicol 50, 2648-2655
8. Yoo WG, Lee JH, Shin Y et al (2014) Antimicrobial peptides in the centipede Scolopendra subspinipes mutilans. Funct Integr Genomics 14, 275-283
9. Lee JH, Kim IW, Kim SH et al (2015) Anticancer Activity of the Antimicrobial Peptide Scolopendrasin VII Derived from the Centipede, Scolopendra subspinipes mutilans. J Microbiol Biotechnol 25, 1275-1280
10. Park YJ, Lee HY, Jung YS et al (2015) Antimicrobial peptide scolopendrasin VII, derived from the centipede Scolopendra subspinipes mutilans, stimulates macrophage chemotaxis via formyl peptide receptor 1. BMB Rep 48, 479-484
11. Agerberth B, Charo J, Werr J et al (2000) The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. Blood 96, 3086-3093
12. Corpet F (1998) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16, 10881-10890
13. Porto BN, Alves LS, Fernández PL et al (2007) Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemotactic receptors. J Biol Chem 282, 24430-24436
14. Spangrude GJ, Sacchi F, Hill HR et al (1985) Inhibition of lymphocyte and neutrophil chemotaxis by pertussis toxin. J Immunol 135, 4135-4143
15. Hampton MB, Kettle AJ and Winterbourn CC (1998) Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 92, 3007-3017
16. Soehnlein O (2009) Direct and alternative antimicrobial mechanisms of neutrophil-derived granule proteins. J Mol Med (Berl) 87, 1157-1164
17. Futosi K, Fodor S and Mocsai A (2013) Neutrophil cell surface receptors and their intracellular signal transduction pathways. Int Immunopharmacol 17, 638-650
18. Liu X, Ma B, Malik AB et al (2012) Bidirectional regulation of neutrophil migration by mitogen-activated protein kinases.
19. Kim D, Kim S, Koh H et al (2001) Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production. FASEB J 15, 1953-1962
20. Wenzel-Seifert K and Seifert R (1993) Cyclosporin H is a potent and selective formyl peptide receptor antagonist. Comparison with N-butryoxy-carbonyl-L-phenylalanly-L-leucyl-L-phenylalanine and cyclosporins. A, B, C, D, and E. J Immunol 150, 4591-4599
21. Bae YS, Song JY, Kim Y et al (2003) Differential activation of formyl peptide receptor signaling by peptide ligands. Mol Pharmacol 64, 841-847
22. Schiffermann E, Showell HV, Corcoran BA et al (1975) The isolation and partial characterization of neutrophil chemotactic factors from Escherichia coli. J Immunol 114, 1831-1837
23. Marasco WA, Phan SH, Krutzsch H et al (1984) Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by Escherichia coli. J Biol Chem 259, 5430-5439
24. Walthier A, Riehemann K and Gerke V (2000) A novel ligand of the formyl peptide receptor, annexin I regulates neutrophil extravasation by interacting with the FPR. Mol Cell 5, 831-840
25. Forsman H and Dahlgren C (2010) The FPR2-induced rise in cytosolic calcium in human neutrophils relies on an emptying of intracellular calcium stores and is inhibited by a gelsolin-derived PIP2-binding peptide. BMC Cell Biol 11, 52
26. Partida-Sánchez S, Cockayne DA, Monard S et al (2001) Cyclic ADP-ribose production by CD38 regulates intracellular calcium release, extracellular calcium influx and chemotaxis in neutrophils and is required for bacterial clearance in vivo. Nat Med 7, 1209-1216
27. Bréchard S and Tschirhart EJ (2008) Regulation of superoxide production in neutrophils: role of calcium influx. J Leukoc Biol 84, 1223-1237
28. Brown AP and Ganey PE (1995) Neutrophil degranulation and superoxide production induced by polychlorinated biphenyls are calcium dependent. Toxicol Appl Pharmacol 131, 198-205
29. Kenakin T (2001) Inverse, protean and ligand-selective agonism: matters of receptor conformation. FASEB J 15, 598-611
30. Lee SK, Kim SD, Kook M et al (2015) Phospholipase D2 drives mortality in sepsis by inhibiting neutrophil extracellular trap formation and down-regulating CXCR2. J Exp Med 212, 1381-1390
31. Bae YS, Bae H, Kim Y et al (2001) Identification of novel chemoattractant peptides for human leukocytes. Blood 97, 2854-2862
32. Grynkiewicz G, Poenie M and Tsien RY (1985) A new generation of Ca2+- indicators with greatly improved fluorescence properties. J Biol Chem 260, 3440-3450
33. Lee HY, Kim SD, Shin JW et al (2010) A pertussis toxin sensitive G-protein-independent pathway is involved in serum amyloid A-induced formyl peptide receptor 2-mediated CCL2 production. Exp Mol Med 42, 302-309
34. Gao J-L, Lee EJ and Murphy PM (1999) Impaired antibacterial host defense in mice lacking the N-formylpeptide receptor. J Exp Med 189, 657-662

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