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Identification of Peptides That Antagonize Formyl Peptide Receptor-Like 1-Mediated Signaling

Yoe-Sik Bae, Ha Young Lee, Eun Jin Jo, Jung Im Kim, Hyun-Kyu Kang, Richard D. Ye, Jong-Young Kwak, and Sung Ho Ryu

Formyl peptide receptor-like 1 (FPRL1) is an important classical chemoattractant receptor that is expressed in phagocytic cells in the peripheral blood and brain. Recently, various novel agonists have been identified from several origins, such as host-derived molecules. Activation of FPRL1 is closely related to inflammatory responses in the host defense mechanism and neurodegenerative disorders. In the present study we identified several novel peptides by screening hexapeptide libraries that inhibit the binding of one of FPRL1’s agonists (Trp-Lys-Tyr-Met-Val-o-Met-CONH₂ (WKYMVm)) to its specific receptor, FPRL1, in RBL-2H3 cells. Among the novel peptides, Trp-Arg-Trp-Trp-Trp-Trp-CONH₂ (WRWWW (WRW₄)) showed the most potent activity in terms of inhibiting WKYMVm binding to FPRL1. We also found that WRW₄ inhibited the activation of FPRL1 by WKYMVm, resulting in the complete inhibition of the intracellular calcium increase, extracellular signal-regulated kinase activation, and chemotactic migration of cells toward WKYMVm. For the receptor specificity of WRW₄ to the FPR family, we observed that WRW₄ specifically inhibited the increase in intracellular calcium by the FPRL1 agonists MMK-1, amyloid β42 (Aβ42) peptide, and F peptide, but not by the FPR agonist, fMLF. To investigate the effect of WRW₄ on endogenous FPRL1 ligand-induced cellular responses, we examined its effect on Aβ42 peptide in human neutrophils. Aβ42 peptide-induced superoxide generation and chemotactic migration of neutrophils were inhibited by WRW₄, which also completely inhibited the internalization of Aβ42 peptide in human macrophages. WRW₄ is the first specific FPRL1 antagonist and is expected to be useful in the study of FPRL1 signaling and in the development of drugs against FPRL1-related diseases.

FPRL1 has important roles in the regulation of immune responses against pathogen infection by modulating the activities of phagocytes (6, 7). In particular, FPRL1 has been reported to mediate phagocyte chemotaxis (8, 9) and to cause superoxide generation and exocytosis in human neutrophils. FPRL1 also has been reported to attenuate HIV-1 infection by desensitizing important chemokine receptors (CCR5 and CXCR4) that act as coreceptors of HIV infection (10). FPRL1, which was found to be highly expressed in mononuclear phagocytes that infiltrated the brain tissues of Alzheimer’s disease patients, also plays a role in the neuronal system. FPRL1 has important implications in several disease states, such as amyloidosis, neurodegenerative disease, and prion disease (11–13).

Recently, several FPRL1 agonists have been identified. They include host-derived agonists, such as LL-37 and a mitochondrial peptide fragment. MYFINILTL (4, 14). One of the important lipid mediators, lipoxin A₄ (LXA₄), also has been reported to bind to FPRL1 (14). Some peptides (T21/DP107, F peptide, and V3 peptide) derived from HIV-1 envelope proteins have been demonstrated to bind FPRL1 (15–17). Trp-Lys-Tyr-Met-O-Met-CONH₂ (WKYMVm), a potent leukocyte-stimulating synthetic peptide (18, 19), was also reported to be a potent peptide ligand for FPRL1 (20).

Although the recruitment of phagocytes into sites of tissue damage and their activation are required for the immune responses, excess recruitment of phagocytes into the infection site causes several adverse effects, for example, tissue damage and inflammatory disease (21). In terms of FPRL1, although the receptor has been believed to play a critical role in the innate immune responses and in some neurodegenerative diseases, FPRL1-specific antagonists have not been reported. To reveal the role of FPRL1 in physiological and pathological conditions, specific FPRL1 antagonists would be highly desirable.
Among the known ligands for FPRL1, WKYMVm, which has potent phagocytic activity with only six amino acids, has several merits with respect to FPRL1 antagonist screening. In this study we identified novel FPRL1 antagonist peptides by screening hexapeptide libraries. We also found that the novel peptides could block FPRL1-induced cellular signaling and cellular responses. We expect that these novel peptides will prove useful in the study of FPRL1-mediated physiological responses.

**Materials and Methods**

**Materials**

F-moc amino acids were obtained from Millipore (Bedford, MA). Rapid-amide resin was obtained from DuPont (Boston, MA). PBMC separation medium (Histopaque-1077) and cytochrome c were purchased from Sigma-Aldrich (St. Louis, MO). Fura-2 penta-acetoxyxymethylester (fura-2/AM) was obtained from Molecular Probes (Eugene, OR). RPMI 1640 was obtained from Invitrogen (Carlsbad, CA), and dialyzed FBS and supplemented bovine serum were purchased from HyClone Laboratories (Logan, UT). Radiiodinated WKYMVm (125I-labeled), which was prepared by conjugation with moniodinated 125I-labeled Bolton and Hunter reagent using a method developed by Nycomed Amersham as described in the product information sheet of the producer (Amersham Pharmacia Biotech), was a gift from Amersham Pharmacia Biotech (Little Chalfont, U.K.). Biotinylated WRWWWW was prepared as described previously (22). Amyloid β42 (Aβ42) was purchased from Bachem Biosciences (King of Prussia, PA). LXA4 was obtained from Biomol (Plymouth Meeting, PA). Anti-phospho-extracellular signal-regulated kinase (ERK) Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti-Aβ42 Ab was obtained from Zymed Laboratories (San Francisco, CA).

**Positional scanning synthetic peptide combinatorial libraries**

The hexapeptide libraries were prepared by the Peptide Library Support Facility of Pohang University of Science and Technology, as described previously (10, 18, 19). Briefly, peptides were synthesized on a RapidAmide support resin and assembled by following the standard F-moc/t-butyl strategy on an acid-labile linker. Peptide compositions were confirmed by amino acid analysis, as previously described (18, 19). Finally, 114 peptide pools (Cys was excluded in the construction of the libraries) were individually dissolved in water at a final concentration of 27 nM/peptide sequence in each peptide pool. Peptides were synthesized using a previously described solid phase method (18).

**Cell culture**

FPRL1-expressing RBL-2H3 cells and vector-transfected RBL-2H3 cells were maintained as previously described (23). Human neutrophils and PBMCs were isolated from healthy donors, as previously described (24). PBMCs were differentiated to macrophages by culturing the cells on four-well Nunc International, Rochester, NY) in RPMI 1640 medium containing 0.1% BSA, 0.01 M HEPES (pH 7.4), and 20 ng/ml monocyte CSF (PeproTech, Rocky Hill, NJ).

**Screening of peptide libraries and ligand binding analysis**

For the initial screening of the positional scanning synthetic peptide combinatorial libraries (PS-SPCLs), we measured the effect of each peptide pool on the binding of 125I-labeled WKYMVm to its specific receptor, FPRL1 in RBL-2H3 cells. Ligand binding analysis was performed as previously described (22, 25). Briefly, FPRL1-expressing RBL-2H3 cells were seeded at 1 × 105 cells/well into a 24-well plate and cultured overnight. After blocking the cells with blocking buffer (33 mM HEPES (pH 7.5) and 0.1% BSA in RPMI 1640) for 2 h, 50 pM labeled WKYMVm was added to the cells in binding buffer (PBS containing 0.1% BSA) in the absence or the presence of unlabeled peptides (final concentration, 0.5 nM/peptide sequence for the initial screening), then incubated for 3 h at 4°C with continuous shaking. The samples were then washed five times with ice-cold binding buffer, and 200 µl of lysis buffer (20 mM Tris (pH 7.5) and 1% Triton X-100) was added to each well. After 20 min at room temperature, the lysates were collected and counted using a gamma counter (25).

**Flow cytometric analysis**

To determine whether WRWWW binds to FPRL1, FPRL1-expressing RBL-2H3 cells were labeled with 10 µM biotin-WRWWW in PBS containing 0.02% sodium azide. The unbound biotin-WRWWW was washed extensively with PBS containing 0.2% BSA and 0.02% sodium azide. Then the cells were incubated with 5 µg/ml streptavidin- FITC at 4°C for 40 min in the dark. Subsequently, cells were washed twice in washing solution (PBS plus BSA and azide) and fixed with 0.2% paraformaldehyde. Fixed cells were analyzed in single laser for FITC with FACS Calibur (BD Biosciences, San Jose, CA).

**Measurement of intracellular calcium concentration ([Ca 2+])**

[Ca 2+]i was determined by Grynkiewicz’s method using fura-2/AM (24). For DNP-human serum albumin (DNP-HSA) stimulation, RBL-2H3 cells were sensitized with 1 µg/ml mouse DNP-specific IgE overnight before fura-2 loading (26).

**Stimulation of cells with peptides for Western blot analysis**

FPRL1-expressing RBL-2H3 cells or isolated human neutrophils (2 × 105) were stimulated with the indicated concentrations of peptides for predetermined times. After stimulation, the cells were washed with serum-free RPMI 1640 and lysed in lysis buffer (20 mM HEPES (pH 7.2), 10% glycerol, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM NaVO4, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM PMSF). Detergent-insoluble materials were pelleted by centrifugation (12,000 × g, 15 min, 4°C), and the soluble supernatant fraction was removed and either stored at −80°C or used immediately. Protein concentrations in the lysates were determined using the Bradford protein assay reagent.

**Electrophoresis and Western blot analysis**

Proteins were separated in 8% SDS-polyacrylamide gel, and the proteins were blotted onto a nitrocellulose membrane, which was then blocked by incubating with Tritis-buffered saline 0.05% Tween 20 containing 5% non-fat dry milk. Subsequently, membranes were incubated with specific Abs and washed with TBS. Ag-Ab complexes were visualized after incubating the membrane with 1/5000 diluted goat anti-rabbit IgG or goat anti-mouse IgG Ab coupled to HRP and were detected by ECL.

**Chemotaxis assay**

Chemotaxis assays were performed using multwell chambers (NeuroProbe, Gaithersburg, MD), as described previously (24, 27). Migrated cells in five randomly chosen high power fields (×400) were then counted.

**Measurement of superoxide generation**

Superoxide anion generation was determined by measuring cytochrome c reduction using a microtiter 96-well plate ELISA reader (EL312e; Bio-Tek Instruments, Winooski, VT) as previously described (24). Superoxide generation was determined for the change in light absorption at 550 nm over 5 min at 1-min intervals.

**Fluorescence confocal microscopy**

Confocal microscopic analysis using anti-Aβ42 Ab was performed as described previously (28). Briefly, human macrophages grown on four-well chamber slides were treated with 10 µM Aβ42 peptide in the absence or the presence of 10 µM WRWWW for different periods at 37°C. The cells were then fixed in 4% paraformaldehyde for 10 min at room temperature, washed with PBS, and incubated with 5% goat serum (Sigma-Aldrich) in PBS containing 0.05% Tween 20 for 1 h to block nonspecific binding and permeabilization. Samples were incubated with anti-Aβ42 Ab for 1 h at room temperature, and slides were washed three times with PBS and incubated with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich; 1/500 dilution in TBS containing 3% BSA) for 30 min. Mounted samples were observed under a laser scanning confocal fluorescence microscope (Zeiss, Oberkochen, Germany).

**Results**

**Identification of peptides that inhibit the binding of WKYMVm to FPRL1**

In this study a total of 114 peptide pools (~47 million peptides) from hexapeptide PS-SPCLs were screened to identify peptides that inhibit the binding of WKYMVm to its cell surface receptor. Fig. 1 shows the results of the initial screening. We observed that amino acids at different positions induced different levels of inhibition of WKYMVm binding to FPRL1. The results shown in Fig. 1 are representative of four independent experiments. The results were very reproducible, because most of the SE values were...
<15% of each mean value. The most active peptides and position were as follows: Lys (K), Arg (R), or Trp (W) in the first position; His (H), Lys (K), or Arg (R) in the second position; Arg (R) or Trp (W) in the third position; Trp (W) in the fourth position; Trp (W) in the fifth position; and Met (M) or Trp (W) in the sixth position of hexapeptides.

Based on the results of the first screening of the peptide libraries, we generated, by reiterative synthesis, four peptide pools, each containing 3 × 3 × 1 × 1 × 1 × 1 = 9 individual hexapeptides. Each peptide mixture contained (D/R/W)(H/K/R)RWWW, (D/R/W)(H/K/R)RRWWW, (D/R/W)(H/K/R)WWW, or (D/R/W)(H/K/R)WWW. The effect of each peptide mixture on the binding of labeled WKYMVm to FPRL1 was examined. A peptide mixture with the sequence (D/R/W)(H/K/R)WWWW showed the greatest activity in terms of competing with WKYMVm binding to FPRL1 (data not shown). The peptide mixture was purified into four fractions by reverse phase HPLC using a C_{18} column (Vydac, Hesperia, CA; 218TP1022, 22 × 250 mm). The effectiveness of the peptides at inhibiting WKYMVm binding to FPRL1 in RBL-2H3 cells was evaluated using the method described for the initial screening. Peptides within the fraction with the greatest activity were identified by mass spectrometry. After confirming the effects of the synthesized single peptides on the inhibition of labeled WKYMVm, we finally identified Trp-Arg-Trp-Trp-Trp-Trp-COH_{2} (WRW4), Arg-His-Trp-Trp-Trp-Trp-COH_{2} (RHW4), Arg-His-Trp-Trp-Trp-Trp-COH_{2} (RHW4), Arg-His-Trp-Trp-Trp-Trp-COH_{2} (RHW4), and Arg-His-Trp-Trp-Trp-Trp-COH_{2} (RHW4) as being the most active peptide in terms of inhibiting the binding of WKYMVm to FPRL1 in RBL-2H3 cells. The inhibitory effects of the four hexapeptides were tested at several concentrations (Fig. 2). The peptide WRW4 was found to most effectively inhibit [\textsuperscript{125}I]-labeled WKYMVm binding. Three other peptides, WRW4, DRW4, and RRW4, also effectively inhibited the binding of labeled WKYMVm (Fig. 2). As a negative control experiment, we confirmed that the random sequence of the hexapeptide (Leu-Phe-Met-Tyr-His-Pro-COH_{2} (LFMYHP)) caused no inhibitory effect at up to 10 \mu M (Fig. 2). The IC_{50} values for the inhibition of 50 pM [\textsuperscript{125}I]-labeled WKYMVm binding to FPRL1 were 0.23 \mu M for WRW4, 3.2 \mu M for RRW4, 2.4 \mu M for RRW4, and 3.4 \mu M for DRW4.

The novel peptide, WRW4, specifically binds to FPRL1

To confirm that WRW4 binds to FPRL1, we synthesized a peptide probe, biotin-WRW4. At first we tested whether the peptide inhibited the binding of [\textsuperscript{125}I]-labeled WKYMVm to FPRL1. The peptide probe biotin-WRW4 was also found to effectively inhibit [\textsuperscript{125}I]-labeled WKYMVm binding (data not shown). Then, we performed a more rigorous experiment using flow cytometric analysis for the specific receptor identification in FPRL1-expressing RBL-2H3 cells. For flow cytometric analysis, vector- or FPRL1-expressing RBL-2H3 cells were labeled with 10 \mu M of the peptide probe, biotin-WRW4, and subsequently with streptavidin-FITC (5 \mu g/ml). Although FPRL1-expressing RBL-2H3 cells are bound by peptide and thereby stained by FITC (Fig. 3A), vector-expressing RBL-2H3 cells are not bound with the peptide (Fig. 3B). Furthermore, staining of FPRL1-expressing RBL-2H3 cells with biotin-WRW4 was almost completely inhibited by the addition of 30 \mu M unlabeled WKYMVm (Fig. 3A). The results indicate that WRW4 specifically binds to FPRL1.

The novel peptide, WRW4, inhibited FPRL1-mediated [Ca\textsuperscript{2+}], increase

The stimulation of FPRL1 by WKYMVm elicited a [Ca\textsuperscript{2+}], increase in FPRL1-expressing RBL-2H3 cells (Fig. 4A). To investigate whether the novel peptides inhibited WKYMVm-induced [Ca\textsuperscript{2+}], increase, we stimulated fura-2-loaded FPRL1-expressing RBL-2H3 cells with various concentrations of WRW4, and subsequently stimulated cells with an effective concentration of WKYMVm. As shown in Fig. 4A, 10 \mu M WRW4 alone did change the intracellular calcium concentration. However, 10 \mu M WRW4 pretreatment before stimulation with 10 nM WKYMVm completely inhibited the [Ca\textsuperscript{2+}] increase induced by WKYMVm in FPRL1-expressing RBL-2H3 cells (Fig. 4A). To confirm specific inhibition by WRW4 on FPRL1-induced signaling, we examined the effect of WRW4 on another extracellular signal-induced [Ca\textsuperscript{2+}], increase. Fce\textsubscript{RI} cross-linking has been reported to induce a [Ca\textsuperscript{2+}], increase in RBL-2H3 cells (26). Stimulation of FPRL1-expressing RBL-2H3 cells (sensitized with 1 \mu g/ml mouse DNP-specific IgE) with 1 \mu g/ml DNP-HSA caused a dramatic increase in [Ca\textsuperscript{2+}], (Fig. 4A). However, pretreatment with 10 \mu M WRW4 before DNP-HSA stimulation did not significantly change the DNP-HSA-induced [Ca\textsuperscript{2+}], increase in RBL-2H3 cells (Fig. 4A). These results indicate that the WRW4-induced inhibition of [Ca\textsuperscript{2+}], increase is a FPRL1-specific event. On examining the concentration-dependency of the WRW4-induced inhibition of
of WRW 4 in terms of ERK phosphorylation by WKYMVm or DNP-H9262.

As shown in Fig. 5A, WRW 4 alone did not affect ERK phosphorylation by WKYMVm. These results strongly suggest that WRW 4 is a specific antagonist of FPRL1.

WRW 4 inhibits FPRL1-mediated ERK phosphorylation

To support our idea that WRW 4 peptide antagonizes FPRL1 signaling, we examined its effect on WKYMVm-induced ERK phosphorylation. Thus, we stimulated FPRL1-expressing RBL-2H3 cells with various concentrations of WRW 4 and measured the ERK phosphorylation level by Western blot with anti-ERK Ab (Fig. 5A). Cells were stimulated with various concentrations of WRW 4 before adding 10 nM WKYMVm or 1 μg/ml DNP-HSA. The results shown are the mean ± SE of four independent experiments (B).

WRW 4 specifically inhibits FPRL1-induced cell signaling in human neutrophils

The effect of WRW 4 on endogenous ligand-induced FPRL1 signaling was investigated in human neutrophils. Aβ42 peptide has previously been reported to act as an endogenous ligand for FPRL1 (12). We found that treatment of human neutrophils with WRW 4 inhibited WRW 4-induced [Ca 2+] i increase by WKYMVm, we observed that WRW 4 inhibited the WKYMVm-mediated process in a concentration-dependent manner, showing maximal inhibition at 10 μM (Fig. 4B); 1 μM WRW 4 inhibited WKYMVm-induced [Ca 2+] i increase by ~55% (Fig. 4B). These results demonstrate that WRW 4 blocks WKYMVm-induced chemotaxis in FPRL1-expressing RBL-2H3 cells in a concentration-dependent manner. However, WRW 4 alone showed no effect on cellular chemotaxis at concentrations of 1 nM to 10 μM in FPRL1-expressing RBL-2H3 cells (Fig. 6A). We then checked the effect of WRW 4 on WKYMVm-induced cellular chemotaxis in FPRL1-expressing RBL-2H3 cells and found that the addition of several concentrations of WRW 4 before chemotaxis assay using 10 nM WKYMVm caused a concentration-dependent inhibition of WKYMVm-induced chemotaxis of the cells (Fig. 6B). WRW 4 (1 μM) blocked ~60% of WKYMVm-induced chemotaxis, and 10 μM WRW 4 almost completely blocked this WKYMVm-induced process (Fig. 6B). The addition of 10 μM of an inactive control peptide, LFMYHP, did not affect WKYMVm-induced chemotaxis (Fig. 6B). These results demonstrate that WRW 4 blocks WKYMVm-induced chemotaxis.

HSA. These results indicate that WRW 4 specifically blocked ERK phosphorylation downstream of FPRL1 by WKYMVm.

WRW 4 inhibits FPRL1-mediated cellular chemotaxis

FPRL1 is a classical chemoattractant receptor, and a previous report demonstrated that WKYMVm induces cellular chemotaxis via FPRL1 (20), which is one of the more important physiological roles of FPRL1. Thus, we investigated the effect of the novel FPRL1 antagonist, WRW 4, on WKYMVm-induced chemotaxis by examining the effect of WRW 4 alone on the chemotaxis of FPRL1-expressing RBL-2H3 cells at various peptide concentrations. As shown in Fig. 6A, WKYMVm caused cellular chemotaxis in FPRL1-expressing RBL-2H3 cells in a concentration-dependent manner. However, WRW 4 alone showed no effect on cellular chemotaxis at concentrations of 1 nM to 10 μM in FPRL1-expressing RBL-2H3 cells (Fig. 6A). We then checked the effect of WRW 4 on WKYMVm-induced cellular chemotaxis in FPRL1-expressing RBL-2H3 cells and found that the addition of several concentrations of WRW 4 before chemotaxis assay using 10 nM WKYMVm caused a concentration-dependent inhibition of WKYMVm-induced chemotaxis of the cells (Fig. 6B). WRW 4 (1 μM) blocked ~60% of WKYMVm-induced chemotaxis, and 10 μM WRW 4 almost completely blocked this WKYMVm-induced process (Fig. 6B). The addition of 10 μM of an inactive control peptide, LFMYHP, did not affect WKYMVm-induced chemotaxis (Fig. 6B). These results demonstrate that WRW 4 blocks WKYMVm-induced chemotaxis.

WRW 4 specifically inhibits FPRL1-induced cell signaling in human neutrophils

The effect of WRW 4 on endogenous ligand-induced FPRL1 signaling was investigated in human neutrophils. Aβ42 peptide has previously been reported to act as an endogenous ligand for FPRL1 (12). We found that treatment of human neutrophils with
that Aβ42 increased superoxide generation in human neutrophils. Moreover, this Aβ42 peptide-induced superoxide-generating activity was concentration dependent and showed a maximal effect at a peptide concentration of 40 μM (Fig. 8A). The addition of WRW4 to human neutrophils at up to 100 μM did not affect superoxide generation (Fig. 8A). However, when neutrophils were preincubated with various concentrations of WRW4, Aβ42 peptide-induced superoxide generation was inhibited in a concentration-dependent manner (Fig. 8B), e.g., 10 μM WRW4 almost completely inhibited superoxide production by Aβ42 peptide (Fig. 8B). As a control experiment we examined the effect of inactive control peptide (LFMYHP) on Aβ42 peptide-induced superoxide generation in human neutrophils. Preincubation with several concentrations of LFMYHP before Aβ42 peptide stimulation did not affect Aβ42 peptide-stimulated superoxide generation in human neutrophils (Fig. 8B).

It has been reported that Aβ42 peptide induces chemotactic migration in human neutrophils via activation of FPRL1 (30). Thus, we examined the effect of Aβ42 on neutrophil chemotaxis and found that Aβ42 peptide induces neutrophil chemotaxis in a concentration-dependent manner (Fig. 8C). Aβ42 (40 μM) induced a 10-fold increase in the number of migrated cells (Fig. 8C). WRW4 alone did not affect neutrophil chemotaxis between 1 and 100 μM (Fig. 8C). Thus, to investigate the effect of WRW4 on Aβ42 peptide-induced neutrophil chemotaxis, we pretreated human neutrophils with several concentrations of WRW4 before the chemotaxis assay with 40 μM Aβ42. Preincubation of neutrophils with WRW4 was found to inhibit Aβ42 peptide-induced neutrophil chemotaxis in a concentration-dependent manner (Fig. 8D), and 10 μM...
WRW\textsuperscript{4} almost completely inhibited the neutrophil chemotaxis induced by A\textbeta 42 peptide (Fig. 8D). Moreover, the control peptide, LFMYHP, did not affect A\textbeta 42 peptide-induced neutrophil chemotaxis (Fig. 8D). These results demonstrate that the selective FPRL1 antagonist, WRW\textsuperscript{4}, inhibits two important A\textbeta 42 peptide-induced cellular responses, namely, superoxide generation and chemotactic migration in human neutrophils.

**WRW\textsuperscript{4} inhibits internalization of A\textbeta 42 peptide in human macrophages**

A\textbeta 42 peptide has been reported to internalize via FPRL1 in human macrophages (28). Because WRW\textsuperscript{4} was found to inhibit the intracellular signaling induced by A\textbeta 42 peptide in human neutrophils, we examined the effect of WRW\textsuperscript{4} on the internalization of A\textbeta 42. When 10 \mu M A\textbeta 42 peptide was incubated in human macrophages, A\textbeta 42 internalization was induced in a time-dependent manner (Fig. 9). A\textbeta 42 peptide internalization began at 5 min, showing maximal internalization at 30 min after incubation (Fig. 9). When human macrophages were pretreated with 10 \mu M WRW\textsuperscript{4} before A\textbeta 42 peptide incubation, we observed that A\textbeta 42 peptide internalization was completely inhibited by WRW\textsuperscript{4} (Fig. 9). This result indicates that the preoccupation of FPRL1 by WRW\textsuperscript{4} inhibits A\textbeta 42 peptide internalization via FPRL1 in human macrophages.

**Discussion**

In this study by screening peptide libraries we identified several hexapeptides that antagonize FPRL1 signaling. W-rich peptides, such as WRW\textsuperscript{4}, RHW\textsuperscript{4}, RRW\textsuperscript{4}, and DRW\textsuperscript{4}, were found to interact directly with FPRL1 and to inhibit the binding of WKYMVm to its cell surface receptor in a concentration-dependent manner. Moreover, WRW\textsuperscript{4} exerted an antagonistic effect on WKYMVm-induced FPRL1 signaling and blocked not only chemotactic migration, but also the superoxide generated by A\textbeta 42 peptide in human neutrophils.

In the process of immunomodulating activity of chemotactants, it is crucial to induce phagocytic cell accumulation into infected tissues (6, 31). Although the excessive recruitment of phagocytic cells causes adverse effects, such as an inflammatory response, few studies have reported the possible negative regulation of chemotactant-induced responses. The direct negative regulation of chemotactant signaling can be induced by interrupting agonist-receptor binding. This study shows that a number of W-rich peptides blocked WKYMVm binding to FPRL1 (Fig. 2). In the experiment using the most efficient peptide, WRW\textsuperscript{4}, we demonstrated that biotin-WRW\textsuperscript{4} binds to FPRL1-expressing RBL-2H3 cells, but not to vector-expressing RBL-2H3 cells (Fig. 3). Taken together the results show that WRW\textsuperscript{4} may bind to FPRL1 and inhibit the binding of WKYMVm to FPRL1. In terms of the peptide, WRW\textsuperscript{4}, we found that it blocked all the tested cellular activities induced by WKYMVm investigated in FPRL1-expressing RBL-2H3 cells. Specifically, WRW\textsuperscript{4} blocked the WKYMVm-induced [Ca\textsuperscript{2+}] increase, ERK activation, and chemotactic migration (Figs. 4–6). Taken together, these findings show that WRW\textsuperscript{4} blocks WKYMVm-initiated FPRL1 signaling by blocking the binding of WKYMVm to the receptor. As FPRL1 is an important
chemoattractant receptor that is involved in the host defense mechanism against pathogen infections, we believe that the W-rich peptides will be useful agents for the development of anti-inflammatory drugs.

Due to the crucial role of FPR family receptors in inflammatory responses, many research groups have tried to identify receptor antagonists for the receptor family. To date, a few antagonists for FPR have been reported (32–34). Two FPR antagonists (t-butyloxy carbonyl-Phe-Leu-Phe-Leu-Phe-OH and i-butyloxy carbonyl-Phe-Leu-Phe-Leu-Phe-OH) have been developed by replacement of the formyl group of iMLF with t-butyloxy carbonyl or i-butyloxy carbonyl (32, 33). A cyclic undecapeptide, cyclosporine H has been developed as a potent and selective FPR antagonist (34). Cyclosporine H has been reported to inhibit FPR-mediated Ca^{2+} mobilization, chemotaxis, and release of proinflammatory mediators (34–36). Even though some FPR-specific antagonists have been developed and investigated for their putative role as therapeutic agents for modulators of inflammatory responses, FPR1-specific antagonists have not been reported until now. In this study we developed several synthetic hexapeptides that act as FPR1 antagonists. We also demonstrated that one of the potent FPR1 antagonists, WRW^4, specifically inhibited the [Ca^{2+}]_i increase induced by all the tested FPR1 agonists (MMK-1, A^42 peptide, and F peptide), but not by iMLF (Fig. 7).

Concerning the efficiency of the novel FPR1 antagonist, WRW^4 inhibited some FPR1 agonist-induced signaling within 1–10 μM (Figs. 4–8). Some chemokine receptor antagonists have been reported. One example is the CCR1 antagonist CP-481,715. This antagonist inhibits the binding of 50 pM 125I-labeled CCL3 in a concentration-dependent manner, showing the complete inhibition at ~10 μM (37). Another CXCR4 antagonist, KRH-1636, inhibits the effective concentration of stromal cell-derived factor 1 (1 μg/ml)-induced Ca^{2+} mobilization at 10 μM (38). As WRW^4 peptide inhibits binding of the homologous ligand by 2-log, 10-fold higher concentrations, this seems to be a weak competitor. Keeping in mind that WRW^4 peptide is the first FPR1 antagonist, it will be used for the development of more improved FPR1-specific antagonists.

In Fig. 7B, we showed that the increase in [Ca^{2+}]_i induced by LXA4 was also inhibited by WRW^4, even though LXA4 has been reported to be a potent anti-inflammatory mediator. Concerning the effect of WRW^4 on the FPR1 ligand peptides or LXA4-induced signaling, WRW^4 inhibited the increase in [Ca^{2+}]_i induced by FPR1 ligand peptides (WKYMVm, F peptide, A^42, and MMK-1) and LXA4. Previously, Chiang et al. (14) demonstrated that two different ligands of FPR1 (LXA4 and MMK-1) could compete with each other. Taken together, it appears that WRW^4 may compete for the binding of FPR1 ligand peptides and LXA4 on FPR1.

Previous reports have shown that inflammation is critically involved in the pathogenesis of Alzheimer’s disease (39). Moreover, Aβ42 peptide is one of the enzymatic cleavage fragments of amyloid precursor protein (40) and has been reported to play a significant role in the proinflammatory responses of systemic amyloidosis, such as Alzheimer’s disease (39, 40). Recently, Aβ42 peptide was found to bind to FPR1 and to modulate the generation of reactive oxygen species and cellular chemotactic migration in human neutrophils via FPR1 (30). Fig. 8 demonstrates that preincubation of human neutrophils with WRW^4 before Aβ42 peptide treatment caused complete inhibition of Aβ42 peptide-induced neutrophil chemotaxis and reactive oxygen species generation. As Aβ42 peptide is critically involved in Alzheimer’s disease, WRW^4 could be used to antagonistically block the action of Aβ42 peptide.

In terms of Alzheimer’s disease, Aβ42 peptide is known to play a central role in mediating neurotoxicity and in the formation of senile plaques (39, 40). It has been reported that mononuclear phagocytes in the brain express FPR1, and that FPR1 gene expression is elevated in CD11b-positive mononuclear phagocytes that infiltrate senile plaques in the brain tissues of Alzheimer’s disease patients (12). Aβ42 peptide also has been reported to increase neurodestructive reactive oxygen species and reactive nitrogen and TNF-α in brain microglia cells and peripheral blood mononuclear phagocytes (41). These molecules are elevated in Alzheimer’s disease (39–41). More recently, Yazawa et al. (28) reported that Aβ42 peptide is internalized via FPR1 and forms fibrillar aggregates in macrophages. In the present study we found that WRW^4 is an FPR1-specific antagonist that blocks the internalization of Aβ42 peptide in human macrophages (Fig. 9). Our results suggest that WRW^4 provides a developmental basis for a putative drug capable of blocking the internalization of Aβ42 peptide and fibrillar formation.

In addition to Aβ42 peptide, several other ligands derived from the HIV-1 envelope domains or host-derived agonists have been reported to bind to FPR1 (14–17). Further studies are needed to evaluate the effects of WRW^4 on these and other FPR1 agonist-related cellular responses and diseases. In summary, we believe that WRW^4, which is the first identified FPR1 antagonist, can be developed as a useful molecule for the study of FPR1 signaling and as a candidate drug for the treatment of several diseases in which FPR1 is known to play a role.

References


FPRL1 ANTAGONISTS


