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Trp-Arg-Trp-Trp-Trp-Trp antagonizes formyl peptide receptor like 2-mediated signaling

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Abstract

Although formyl peptide receptor like 2 (FPRL2) has been regarded as an important classical chemoattractant receptor, its functional role and signaling pathway have not been fully investigated, because of the lack of its specific ligand. Recently F2L, a heme-binding protein fragment peptide, has been reported as an FPRL2-selective endogenous agonist. In the present study, we examined the effect of Trp-Arg-Trp-Trp-Trp-Trp-$CONH_2$ (WRWWWW, WRW^4), on F2L-induced cell signaling. WRW^4 inhibited the activation of FPRL2 by F2L, resulting in the complete inhibition of intracellular calcium increase and chemotactic migration induced by F2L. WRW^4 also completely inhibited F2L-induced NF-κB activation in FPRL2-transfected HEK293 cells. WRW^4 specifically inhibited F2L-induced intracellular calcium increase and chemotactic migration in mature monocyte-derived dendritic cells, which express FPRL2 but not the other FPR family. Taken together, WRW^4 is the first FPRL2 antagonist and is expected to be useful in the study of FPRL2 signaling and in development of drugs against FPRL2-related cellular responses.

Keywords: Formyl peptide receptor like 2; F2L; Antagonist; Intracellular calcium increase; Chemotaxis; Monocytes

Formyl peptide receptor like 2 (FPRL2), which was initially cloned as a formyl peptide receptor (FPR) homologue by low stringency hybridization with the human FPR cDNA probe [1], is one of the classic chemoattractant receptors, i.e., a G protein-coupled seven transmembrane receptors. Previous report suggested the role of FPRL2 in the regulation of phagocytic cellular responses such as chemotactic migration [2]. Even though FPRL2 has been regarded as an important chemoattractant receptor, the lack of its specific agonist has made limited the study on the cellular functioning.

Recently, several FPRL2 agonists have been identified. They include host-derived agonist, F2L, a heme-binding protein fragment [3]. One bacterial peptide, Hp (2–20), derived from *Helicobacter pylori* has been demonstrated to bind FPRL2 [4]. Two potent leukocyte stimulating synthetic peptides, Trp-Lys-Tyr-Met-Val-D-Met-$CONH_2$ (WKYMVm) [5], and Trp-Lys-Tyr-Met-Val-Met-$CONH_2$ (WKYMVM) [6], were also reported to be potent peptide ligands for FPRL2 [2]. Since FPRL2 is an important mediator of phagocyte recruitment into sites of tissue damage, a molecule that inhibits the FPRL2-mediated signaling is essentially required. Putative FPRL2 antagonist also will be useful for the revelation of the role of FPRL2 in physiological and pathological conditions.

In this study, we examined the effect of a previously reported FPRL1 antagonist, Trp-Arg-Trp-Trp-Trp-Trp-$CONH_2$ (WRWWWW, WRW^4) [7], on FPRL2-induced cell signaling and cellular responses.

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Materials and methods

Materials. Peripheral blood mononuclear cell separation medium (Histopaque-1077) and formyl-Met-Leu-Phe (fMLF) were from Sigma (St. Louis, MO). Fura-2 pentaacetoxymethylester (fura-2/AM) was from Molecular Probes (Eugene, OR). RPMI 1640 medium and dialyzed fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). Acetylated F2L and MMK-1 were synthesized as reported [3,8] from Anygen (Gwangju, Korea). WRW^4 and WKYMVm were synthesized by A & Pep. (Yeongi, Korea).

Isolation of human peripheral blood leukocytes. Peripheral blood was collected from healthy donors, and peripheral blood mononuclear cells were isolated by separation on a Histopaque-1077 gradient. After two washings with Hank's buffered saline solution, without Ca^{2+} and Mg^{2+}, peripheral blood mononuclear cells were suspended in RPMI 1640 medium containing 10% FBS and incubated for 60 min at 37 °C to allow monocytes attachment to the culture dish. Attached monocytes were then collected as described previously [9]. Human neutrophils were isolated according to the standard procedures of dextran sedimentation, hypotonic lysis of erythrocytes, and a lymphocyte separation medium gradient as described previously [10]. The isolated human leukocytes were then used promptly.

Generation of human monocyte-derived dendritic cells. Human monocyte-derived dendritic cells (MoDCs) were generated as described previously [11]. Peripheral blood monocytes were differentiated to MoDCs by culturing the cells in 6-well plate in 2 ml of complete medium (RPMI 1640 medium supplemented with 10% FBS) supplemented with recombinant human GM-CSF (10 ng/ml; Pierce Endogen, Rockford, IL) and recombinant human IL-4 (10 ng/ml; Pierce Endogen, Rockford, IL) [11]. All cultures were incubated at 37 °C in 5% humidified CO_2. After 7 days of culture, MoDCs were stimulated with 100 ng/ml of lipopolysaccharide for 48 h for maturation.

Transient transfection of FPRL2. HA-tagged human FPRL2 cDNA was purchased from UMR cDNA Resource Center (Rolla, Mo). Transfections were performed using LipofectAMINE reagents (Invitrogen Corporation) according to the manufacturer's instructions. HEK293 cells were harvested 48 h after transfection and expression of HA-tagged FPRL2 protein was examined by Western blotting using monoclonal anti-HA antibody (Sigma) (data not shown).

Measurement of intracellular calcium concentration. Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined by Grynkiewicz's method using fura-2/AM [9]. Briefly, prepared cells were incubated with 3 μM fura-2/AM at 37 °C for 50 min in serum free RPMI 1640 medium with continuous stirring. 2×10^6 cells were aliquoted for each assay into 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). Fluorescence was measured at 500 nm at excitation wavelengths of 340 and 380 nm.

Chemotaxis assay. Chemotaxis assays were performed using multiwell chambers (Neuroprobe, Gaithersburg, MD) as described previously [9]. Briefly, polycarbonate filter (5 μm pore size) was placed on a 96-well chamber containing different concentrations of peptides. Freshly isolated human monocytes or mature MoDCs were suspended in RPMI 1640 medium at a concentration of 1×10^6 cells/ml, and 25 μl of the cell suspension were placed onto the upper well of the chamber. After incubation for 2 h at 37 °C, non-migrating cells were removed by scraping, and cells that migrated across the filter were dehydrated, fixed, and stained with hematoxylin (Sigma, St. Louis, MO). The stained cells in five randomly chosen high power fields (HPF, 400×) were then counted for each well.

Luciferase assay. The pGL2-Basic vector containing a polyadenylation signal upstream from the luciferase gene was used to construct expression vectors by subcloning PCR-amplified DNA of NF-κB promoter into the *Sac*I/*Hin*dIII site of the pGL2-Basic vector. Transfection of NF-κB promoter-luc (10 μg/assay) was performed on HEK293 cells using LipofectAMINE. Two days after transfection, the cells were stimulated with several peptides for 1 h. Cells were lyzed with lysis buffer (20 mM Tris–HCl, pH 7.8, 1% Triton X-100, 150 mM NaCl, and 2 mM DTT). The cell lysate 5 μl was mixed with luciferase activity assay reagent 25 μl and luminescence produced for 5 s was measured using luminoskan (labsystems).

Results

F2L induces $[Ca^{2+}]_i$ increase in human monocytes but not in neutrophils

Previously F2L has been identified as an endogenous ligand for FPRL2 [3]. To confirm whether F2L stimulates FPRL2, resulting in $[Ca^{2+}]_i$ increase, we examined the effect of F2L on calcium signaling in human monocytes which express FPRL2 [12]. As expected, 20 μM of F2L stimulated $[Ca^{2+}]_i$ increase in human monocytes (Fig. 1A). As positive control fMLF (a selective agonist for FPR) [13] and MMK-1 (a selective agonist for FPRL1) [14] also stimulated human monocytes, eliciting $[Ca^{2+}]_i$ increase (Fig. 1A). We also tested the effect of the three

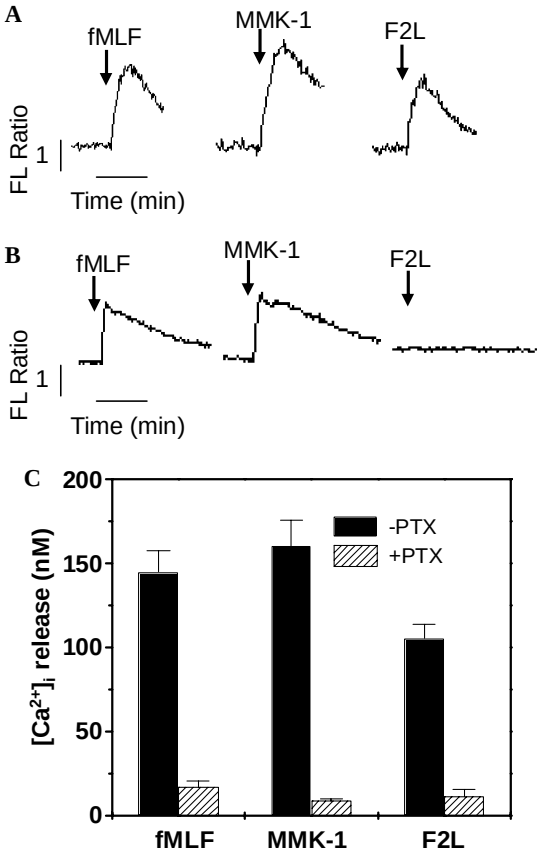

Fig. 1. Effect F2L on $[Ca^{2+}]_i$ rise in human monocytes and neutrophils. Freshly isolated human monocytes (A), or neutrophils (B) were stimulated with fMLF (1 μM), MMK-1 (1 μM), or F2L (20 μM). The changes in 340/80 nm were monitored. The results are representative of three independent experiments (A,B). Monocytes were preincubated with or without 100 ng/ml of PTX for 24 h, prior to fura-2/AM loading (C). Fura-2/AM loaded cells were stimulated with fMLF (1 μM), MMK-1 (1 μM), or F2L (20 μM). The changes in 340/80 nm were monitored. The peak level of $[Ca^{2+}]_i$ was recorded. Results are presented as means ± SE of three independent experiments, performed in duplicate (C).

peptides on $[Ca^{2+}]_i$ increase in human neutrophils. As shown in Fig. 1B, both of fMLF and MMK-1 stimulated $[Ca^{2+}]_i$ increase in human neutrophils. F2L, however, did not affect on $[Ca^{2+}]_i$ increase in human neutrophils (Fig. 1B). Since human monocytes but not neutrophils have been reported to express FPRL2, the results support the previous report that F2L is an FPRL2-selective agonist. We also examined the effect of pertussis toxin (PTX) on the F2L-induced $[Ca^{2+}]_i$ increase in human monocytes. As shown in Fig. 1C, preincubation of human monocytes with 100 ng/ml of PTX for 24 h almost completely inhibited F2L-induced $[Ca^{2+}]_i$ increase. fMLF- and MMK-1-induced $[Ca^{2+}]_i$ increases were also completely inhibited by PTX (Fig. 1C). The results suggest that F2L stimulates $[Ca^{2+}]_i$ increase via the activation of PTX-sensitive Gi protein(s) in human monocytes.

WRW^4 inhibits F2L-induced $[Ca^{2+}]_i$ increase

The stimulation of human monocytes by F2L elicited a $[Ca^{2+}]_i$ increase (Figs. 1A and 2A). To investigate whether WRW^4 inhibits F2L-induced $[Ca^{2+}]_i$ increase, we stimulated fura-2 loaded human monocytes with various concentrations of WRW^4, and subsequently stimulated with an effective concentration of F2L (20 μM). As shown in Fig. 2A, 10 μM WRW^4 alone did not change the intracellular calcium concentration in human monocytes. However, 10 μM WRW^4 pretreatment prior to stimulation with 20 μM F2L completely inhibited the $[Ca^{2+}]_i$ increase induced by F2L in human monocytes (Fig. 2A). To confirm specific inhibition by WRW^4 on F2L-induced signaling, we examined the effect of WRW^4 on another extracellular signal-induced $[Ca^{2+}]_i$ increase. Leukotriene B4 (LTB4) has been reported to induce a $[Ca^{2+}]_i$ increase in human monocytes [15]. Stimulation of human monocytes with 1 μM LTB4 caused a dramatic increase in $[Ca^{2+}]_i$ (Fig. 2A). However, pretreatment with 10 μM WRW^4 prior to LTB4 stimulation did not significantly change the LTB4-induced $[Ca^{2+}]_i$ increase in human monocytes (Fig. 2A). These results indicate that the WRW^4-induced inhibition of $[Ca^{2+}]_i$ increase is an FPRL2-specific event. As we already reported, WRW^4 also completely inhibited WKYMVm-induced $[Ca^{2+}]_i$ increase (Fig. 2A). On examining the concentration-dependency of the WRW^4-induced inhibition of $[Ca^{2+}]_i$ increase by F2L, we observed that WRW^4 inhibited the F2L-mediated process in a concentration-dependent manner, showing maximal inhibition at 10 μM (Fig. 2B); 1 μM WRW^4 inhibited F2L-induced $[Ca^{2+}]_i$ increase by around 70% (Fig. 2B). These results strongly suggest that WRW^4 is a specific antagonist of FPRL2.

WRW^4 inhibits F2L-induced cellular chemotaxis

F2L has been reported to induce chemotactic migration in human monocyte and MoDCs [3]. We also examined the effect of F2L on the migration of human monocytes. F2L

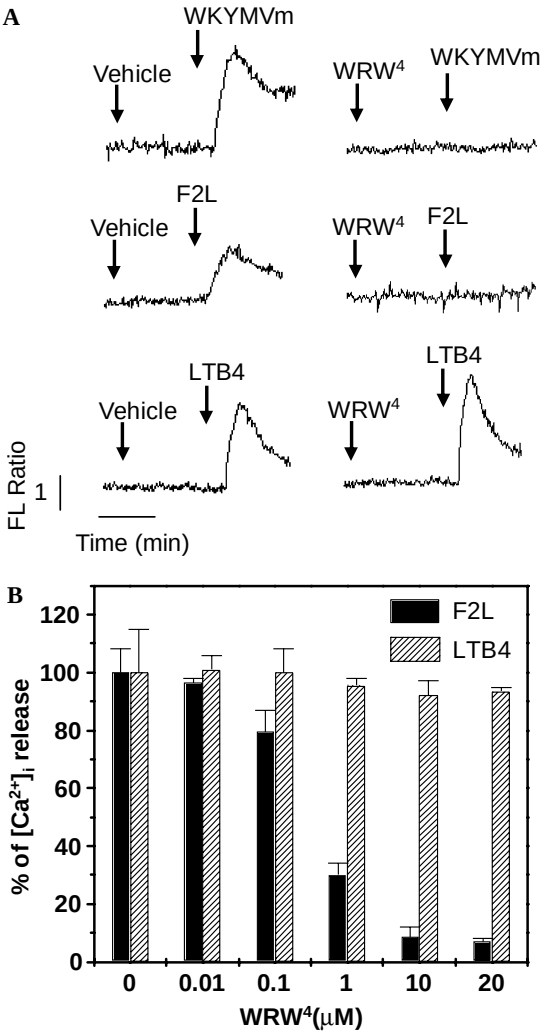

Fig. 2. Effect of WRW^4 on the F2L-induced $[Ca^{2+}]_i$ rise in human monocytes. Freshly isolated human monocytes were stimulated with vehicle or WRW^4 (10 μM) and then with WKYMVm (10 nM), F2L (20 μM), or with LTB4 (1 μM). The changes in 340/380 nm were monitored. The results are representative of three independent experiments (A). Cells were stimulated with various concentrations of WRW^4 prior to adding 20 μM of F2L or 1 μM of LTB4. The results shown are means ± SE of four independent experiments (B).

induced a chemotactic migration in a 10–20 μM in human monocytes (Fig. 3A). F2L-mediated chemotactic activity at a concentration of 10 μM peptide is about 81% of that of the 100 nM WKYMVm-induced one in human monocytes. In five experiments with differently prepared human peripheral monocytes F2L induced chemotactic activity in a similar pattern. We examined the effect of PTX on the F2L-induced chemotaxis by human monocytes. When monocytes were preincubated with 100 ng/ml of PTX for 24 h prior to the chemotaxis assay, the number of cells migrating toward F2L (20 μM) was reduced by 93% in comparison to the numbers of cells not treated with PTX (Fig. 3B). To distinguish the F2L-induced chemotaxis and chemokinesis, we performed a checkerboard analysis as described previously [16]. A gradually increasing concentration gradient of F2L between lower chamber and upper

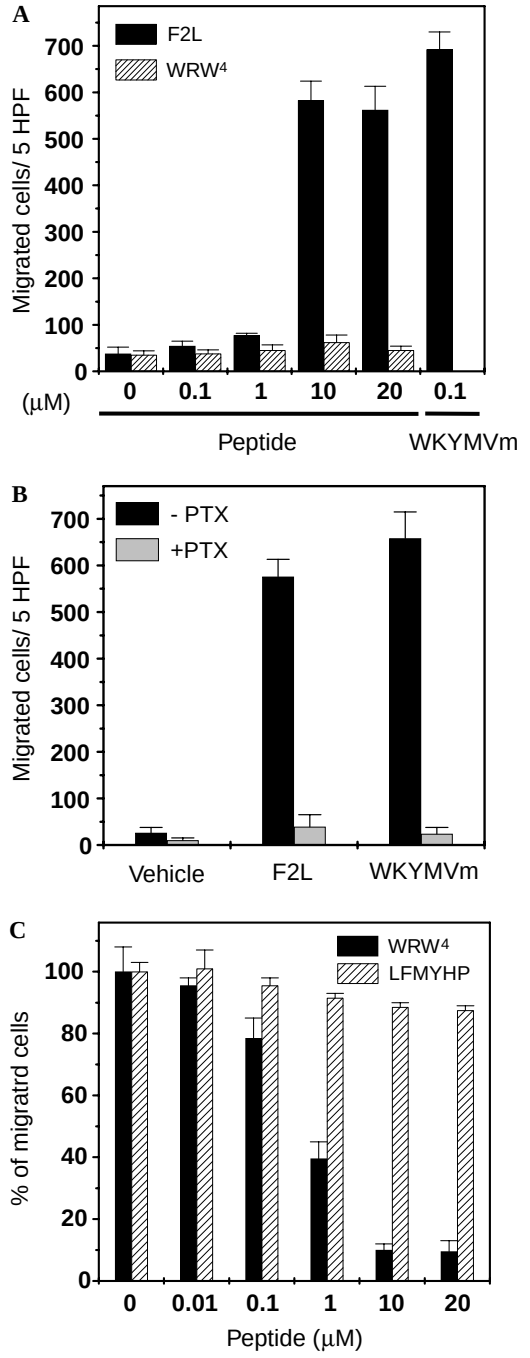

Fig. 3. Effect of WRW^4 on the F2L-induced monocyte chemotaxis. Human monocytes (1×10^6 cells/ml of RPMI 1640 medium) were added to the upper wells of a 96-well chemotaxis chamber for 2 h at 37 °C. Various concentrations of WRW^4 or F2L were used in the assays (A). Monocytes were preincubated with or without 100 ng/ml of PTX for 24 h prior to chemotaxis assay with 20 μM of F2L or 100 nM of WKYMVm (B). Several concentrations of WRW^4 or LFMYHP were added for 5 min prior to the chemotaxis assay using 20 μM of F2L (C). Numbers of migrated cells were determined by counting in a high power field (400×). Results are presented as means ± SE of three independent experiments each performed in duplicate.

chamber induced significant migration of monocytes to the peptide (data not shown). This implies F2L induces chemotaxis of the cells. These results, therefore, indicate that F2L stimulates the chemotaxis of human monocytes in a PTX-sensitive G-protein.

We then, investigated the effect of WRW^4 on F2L-induced chemotaxis. At first we examined the effect of WRW^4 alone on the chemotaxis of human monocytes at various peptide concentrations. As shown in Fig. 3A, WRW^4 alone showed no effect on cellular chemotaxis at concentrations of 0.1–20 μM in human monocytes (Fig. 3A). We, then, checked the effect of WRW^4 on F2L-induced cellular chemotaxis in human monocytes, and found that the addition of several concentrations of WRW^4 prior to chemotaxis assay using 20 μM F2L caused a concentration-dependent inhibition of F2L-induced chemotaxis of the cells (Fig. 3C). 1 μM WRW^4 blocked ca. 60% of F2L-induced chemotaxis and 10 μM WRW^4 almost completely blocked this F2L-induced process (Fig. 3C). The addition of various concentrations of an inactive control peptide Leu-Phe-Met-Tyr-His-Phe-$CONH_2$ (LFMYHP) [9] did not affect F2L-induced chemotaxis (Fig. 3C). These results demonstrate that WRW^4 blocks F2L-induced chemotaxis.

WRW^4 inhibits FPRL2-mediated NF-κB activation

To support our notion that WRW^4 peptide antagonizes FPRL2 signaling, we examined its effect on F2L-induced NF-κB activation. At first we confirmed that F2L (20 μM) did not stimulate wild type HEK293 cells (data not shown), which does not express FPRL2. Thus, we stimulated FPRL2/NF-κB luciferase-transfected HEK293 cells with 10 μM of WRW^4 and measured the luciferase activity using lucigenin as a substrate. As shown in Fig. 4, WRW^4

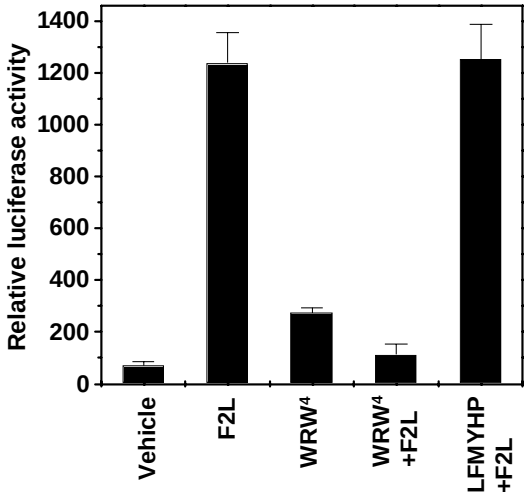

Fig. 4. Effect of WRW^4 on the F2L-induced NF-κB activity. FPRL2 cDNA and NF-κB-luc cDNA were co-transfected into HEK293 cells. WRW^4 (10 μM) or LFMYHP (10 μM) were added for 5 min prior to stimulating cells with vehicle or F2L (20 μM). Luciferase activities were measured in cell lysates using luciferase reporter gene assays. Emitted luminescence is expressed in relative luminescent units. The data shown represent means ± SE from two separate experiments performed in duplicate.

alone did not affect NF-κB activity in the cells. However, when we stimulated FPRL2/NF-κB luciferase-transfected HEK293 cells with 20 μM of F2L for 1 h, we observed a dramatic increase in the luciferase activity (Fig. 4). Moreover, pretreatment with 10 μM of WRW^4 inhibited F2L-elicited NF-κB activity (Fig. 4). And, a pretreatment with an inactive control peptide LFMYHP (10 μM) did not inhibit NF-κB activity induced by F2L (20 μM) (Fig. 4). To demonstrate that WRW^4 does not inhibit NF-κB activity induced by non-FPRL2 agonist, we performed control experiment using ATP. Preincubation of FPRL2/NF-κB luciferase-expressing HEK293 cells with 10 μM of WRW^4 prior to the stimulation with ATP did not affect on the ATP-induced NF-κB activation (data not shown). These results indicate that WRW^4 specifically blocks NF-κB activity downstream of FPRL2 by F2L.

WRW^4 inhibits F2L-induced $[Ca^{2+}]_i$ increase and chemotactic migration in mature MoDCs

Since it has been reported that mature MoDCs express only FPRL2 but not the other FPR family (FPR and FPRL1) [11,12], we examined the effect of WRW^4 on F2L-induced signaling in mature MoDCs. The stimulation of mature human MoDCs by F2L elicited a $[Ca^{2+}]_i$ increase (Fig. 5A). To investigate whether WRW^4 inhibits F2L-induced $[Ca^{2+}]_i$ increase, we stimulated fura-2 loaded mature MoDCs with 10 μM of WRW^4, and subsequently stimulated with an effective concentration of F2L (20 μM). As shown in Fig. 5A, 10 μM WRW^4 alone did change the intracellular calcium concentration. However, 10 μM WRW^4 pretreatment prior to stimulation with 20 μM F2L completely inhibited the $[Ca^{2+}]_i$ increase induced by F2L in mature MoDCs (Fig. 5A). However, pretreatment with 10 μM WRW^4 prior to ATP (500 μM) stimulation did not significantly change the ATP-induced $[Ca^{2+}]_i$ increase in mature MoDCs (Fig. 5A).

We examined the effect of F2L on the migration of mature MoDCs. F2L induced a chemotactic migration at 10–20 μM in mature MoDCs (Fig. 5B). We then, investigated the effect of WRW^4 on F2L-induced chemotaxis. As shown in Fig. 5B, WRW^4 alone showed no effect on cellular chemotaxis at concentration at 10 μM in mature MoDCs. We then, checked the effect of WRW^4 on F2L-induced cellular chemotaxis in mature MoDCs, and found that the addition of 10 μM of WRW^4 prior to chemotaxis assay using 10–20 μM of F2L caused almost complete inhibition of F2L-induced chemotaxis of the cells (Fig. 5B). These results demonstrate that WRW^4 blocks F2L-induced chemotaxis in mature MoDCs.

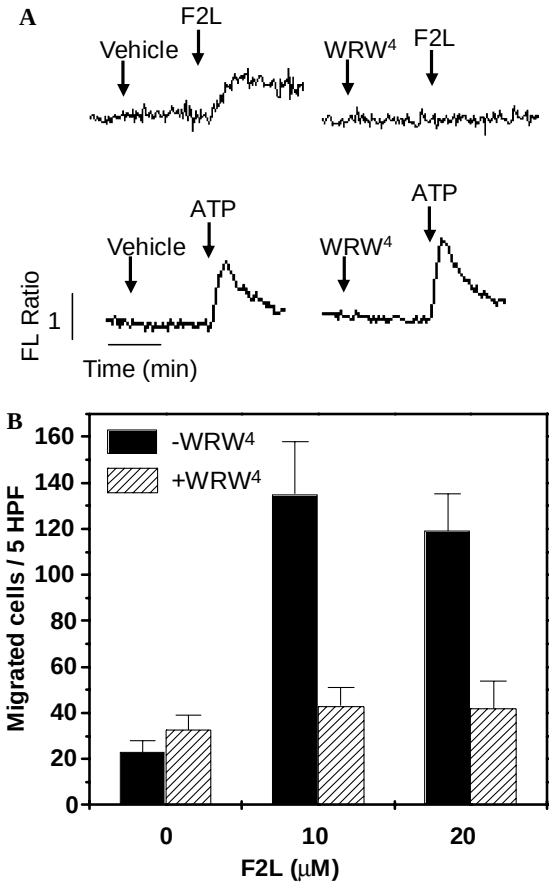

Fig. 5. Effect of WRW^4 on the F2L-induced calcium signaling and chemotaxis in mature MoDCs. Mature MoDCs were stimulated with vehicle or WRW^4 (10 μM) and then with F2L (20 μM), or ATP (500 μM). The changes in 340/380 nm were monitored. The results are representative of three independent experiments (A). Mature MoDCs (1×10^6 cells/ml of RPMI 1640 medium) were added to the upper wells of a 96-well chemotaxis chamber for 2 h at 37 °C. Vehicle or 10 μM of WRW^4 was added prior to the chemotaxis assay using F2L (10 μM or 20 μM) (B). Numbers of migrated cells were determined by counting in a high power field (400×). Results are presented as means ± SE of three independent experiments each performed in duplicate.

Discussion

In the process of immuno-modulating activity of chemoattractants, it is crucial to induce phagocytic cells accumulation into infected tissues [17]. Since the excessive recruitment of phagocytic cells causes adverse effects, such as inflammatory response, the development of antagonists for chemoattractant receptors is essentially required. This study shows that WRW^4 blocks F2L-induced $[Ca^{2+}]_i$ increase, chemotactic migration, and NF-κB activation (Figs. 2–4). Taken together, these findings show that WRW^4 inhibits F2L-initiated FPRL2-signaling by acting on FPRL2.

Because FPR family receptors have crucial roles 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 [18–20], including *t*BOC-Phe-Leu-Phe-Leu-Phe-OH and *i*BOC-Phe-Leu-Phe-Leu-Phe-OH. On the antagonist for FPRL1, only WRW^4 has been reported until now [7]. Even tough some FPR- or FPRL1-specific antagonists have been developed and investigated for their putative role as therapeutic agents for modulators of inflammatory responses, FPRL2-specific antagonists have not been reported until

now. In this study we found that WRW^4 acts as an antagonist not only for FPRL1 but also for FPRL2.

Previous reports have shown that the expression of FPRL2 is maintained throughout the maturation of DCs [11,12]. The result suggests that FPRL2 may be involved in the migration of mature DCs into specific anatomical site [11,12]. Reminding that mature DC migration into lymph node is important for the induction of antigen-specific immune response [21], and F2L can induce mature DC migration, our finding that WRW^4 inhibits F2L-induced mature DC migration suggests that WRW^4 can be used as a negative regulator of DC-mediated induction of antigen-specific immune responses.

Further studies are needed to evaluate the effect of WRW^4 on FPRL2 agonist-related cellular responses and diseases. In summary, we believe that WRW^4, which is the first identified FPRL2 antagonist, can be developed as a useful molecule for the study of FPRL2 signaling and as a candidate drug for the treatment of several diseases in which FPRL2 is known to play a role.

Acknowledgments

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