The Lipidated Peptidomimetic Lau-((*S*)-Aoc)-(Lys-βNphe)$_6$-NH$_2$ Is a Novel Formyl Peptide Receptor 2 Agonist That Activates Both Human and Mouse Neutrophil NADPH Oxidase*[S]

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Neutrophils expressing formyl peptide receptor 2 (FPR2) play key roles in host defense, immune regulation, and resolution of inflammation. Consequently, the search for FPR2-specific modulators has attracted much attention due to its therapeutic potential. Earlier described agonists for this receptor display potent activity for the human receptor (FPR2) but low activity for the mouse receptor orthologue (Fpr2), rendering them inapplicable in murine models of human disease. Here we describe a novel FPR2 agonist, the proteolytically stable α-peptide/β-peptoid hybrid Lau-((*S*)-Aoc)-(Lys-βNphe)$_6$-NH$_2$ (F2M2), showing comparable potency in activating human and mouse neutrophils by inducing a rise in intracellular Ca^{2+} concentration and assembly of the superoxide-generating NADPH oxidase. This FPR2/Fpr2 agonist contains a headgroup consisting of a 2-aminooctanoic acid (Aoc) residue acylated with lauric acid (C$_{12}$ fatty acid), which is linked to a peptide/peptoid repeat ((Lys-βNphe)$_6$-NH$_2$). Both the fatty acid moiety and the (*S*)-Aoc residue were required for FPR2/Fpr2 activation. This type of proteolytically stable FPR2-specific peptidomimetics may serve as valuable tools for future analysis of FPR2 signaling as well as for development of prophylactic immunomodulatory therapy. This novel class of cross-species FPR2/Fpr2 agonists should enable translation of results obtained with mouse neutrophils (and disease models) into enhanced understanding of human inflammatory and immune diseases.

The molecular basis for directional neutrophil migration toward inflammatory sites is their ability to sense "danger signals" produced by microorganisms (*i.e.* pathogen-associated molecular patterns) and damaged host cells or tissues (*i.e.* damage-associated molecular patterns) (1). Human neutrophils express two members of the formyl peptide receptor family (FPR1[3] and FPR2), belonging to the family of G-protein-coupled receptors (GPCRs), which recognize pathogen-associated molecular patterns in the form of peptides with an N-terminal formyl-methionine residue, originating from bacterial (and mitochondrial) protein synthesis (2, 3). In addition, human neutrophils express GPCRs that recognize endogenous chemoattractants and damage-associated molecular patterns, including the split product of complement component C5 (C5a, recognized by C5aR), leukotriene LTB$_4$ (recognized by BLT1), the chemokine IL-8 (CXCL8, recognized by CXCR1 and CXCR2), platelet-activating factor (PAF, recognized by PAFR), and the nucleotide ATP (recognized by P2Y$_2$R) (4, 5). Although the two human neutrophil FPRs, FPR1 and FPR2, display significant sequence similarity, they have distinct, albeit partially overlapping, ligand recognition profiles (2, 6, 7).

The mouse genome contains at least eight mouse Fprs, among which Fpr1 and Fpr2 are regarded as the orthologues of the receptors expressed in human neutrophils (8, 9). Studies using mice deficient in Fpr1 or Fpr2 have demonstrated roles of these receptors not only in host defense but also in immune regulation and in the resolution of inflammation (10–12). The therapeutic potential of targeting these receptors in inflammatory/infectious conditions, such as atherosclerosis, cancer, neurodegenerative diseases, and sepsis (see a recent review (13)), justifies the search for receptor-specific ligands that can function as agonists, antagonists, or allosteric modulators. However, a direct translation of knowledge between humans and

[3] The abbreviations used are: FPR, formyl peptide receptor; βNphe, *N*-phenylmethyl-β-alanine; Aoc, 2-aminooctanoic acid; GPCR, G-protein-coupled receptor; KRG, Krebs-Ringer phosphate buffer supplemented with glucose; Lat A, latrunculin A; Lau, lauroyl; PAF, platelet-activating factor; PAFR, PAF receptor; PMA, phorbol 12-myristate 13-acetate; fMLF, formylmethionylleucylphenylalanine; PBP$_{10}$, RhB-QRLFQVKGRR; F2M2, Lau-((*S*)-Aoc)-(Lys-βNphe)$_6$-NH$_2$.

A

W K Y M V M
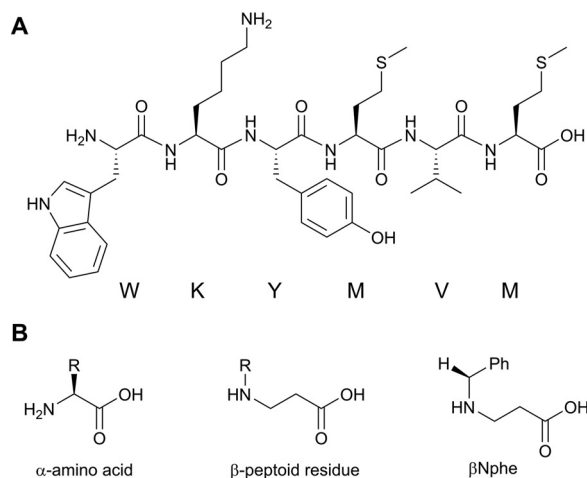
B

α-amino acid   β-peptoid residue   βNphe

FIGURE 1. **Structures of the FPR2 agonist WKYMVM and peptidomimetic residues.** *A*, molecular structure of FPR2-specific peptide agonist WKYMVM. *B*, general formulas for α-amino acids and β-peptoid units that contain an additional carbon atom in the β-position (as compared with α-amino acids). In β-peptoid units, the side chain (*R*) is bound to the nitrogen atom rather than to the α-carbon. βNphe is the β-peptoid analogue of phenylalanine used in the synthesis of peptidomimetics F2M2–F2M10 and F2M12–F2M18 (exact structures are found in Fig. 2).

mice is not possible, due to the fact that there are many species-associated differences in both agonist and antagonist recognition profiles by the receptors. This is clearly illustrated by the fact that fMLF, the potent prototype FPR1 agonist, is a very weak agonist for Fpr1 (14). Similarly, the potent FPR2 agonist WKYMVM (Fig. 1*A*) is a weak agonist for Fpr2 (9, 15). To our knowledge, the short formyl peptides formyl-MIVIL and formyl-MIFL from *Listeria monocytogenes* and *Staphylococcus aureus*, respectively, are the most potent Fpr1 agonists currently identified (16), whereas potent Fpr2-selective ligands remain to be identified. By contrast, several FPR2-selective ligands have successfully been described and characterized. Potent and stable modulators targeting both human and mouse receptors (especially FPR2 and Fpr2) thus remain to be identified. Such compounds would facilitate Fpr-based studies in mouse models of disease, important for understanding the precise roles of FPRs in health and disease, and for the validation of potential drug lead candidates.

A major obstacle for therapeutic use of peptides is their inherent susceptibility to degradation by endogenous proteases, resulting in low bioavailability. Thus, investigation of proteolytically stable peptidomimetics, partly composed of unnatural residues, appears advantageous. Stable peptidomimetics belonging to the class of α-peptide/β-peptoid hybrids, displaying a design with alternating cationic α-amino acids and lipophilic peptoid residues, have been shown to imitate the biological effects of natural antimicrobial peptides (*e.g.* antimicrobial activity, antibiofilm activity, and immunomodulatory activity) (17–21). We have previously identified a novel class of FPR2 antagonists by screening an array of peptidomimetics comprising several subclasses of α-peptide/β-peptoid hybrids (see Fig. 1*B* for generic structures), using GPCR-dependent neutrophil production of superoxide as a read-out (22). Among these compounds, the most promising FPR2-interacting peptidomimetic, palmitoyl-(Lys-βNSpe)$_6$-NH$_2$ (F2M1 in Fig. 2), was found to inhibit neutrophil func-

F2M1: R = Pam; R' = Me
F2M17: R = Lau; R' = H
F2M18: R = Ac; R' = H

F2M2: R = Lau ; n = 3
F2M3: R = Oct ; n = 3
F2M4: R = Dec ; n = 3
F2M5: R = Myr ; n = 3
F2M6: R = Pam ; n = 3
F2M7: R = Lau ; n = 1
F2M8: R = Lau ; n = 5
F2M9: R = Lau ; n = 7
F2M16: R = Ac ; n = 3

F2M10: R = Lau

F2M11: R = Lau

F2M12: R = R' = F;
F2M13: R = F ; R' = H
F2M14: R = Me ; R' = H

F2M15

Abbreviations used:
βNphe = N-Phenylmethyl-β-Ala
Ac = acetyl
Dec = decanoyl
Lau = lauroyl
Me = methyl
Myr = myristoyl
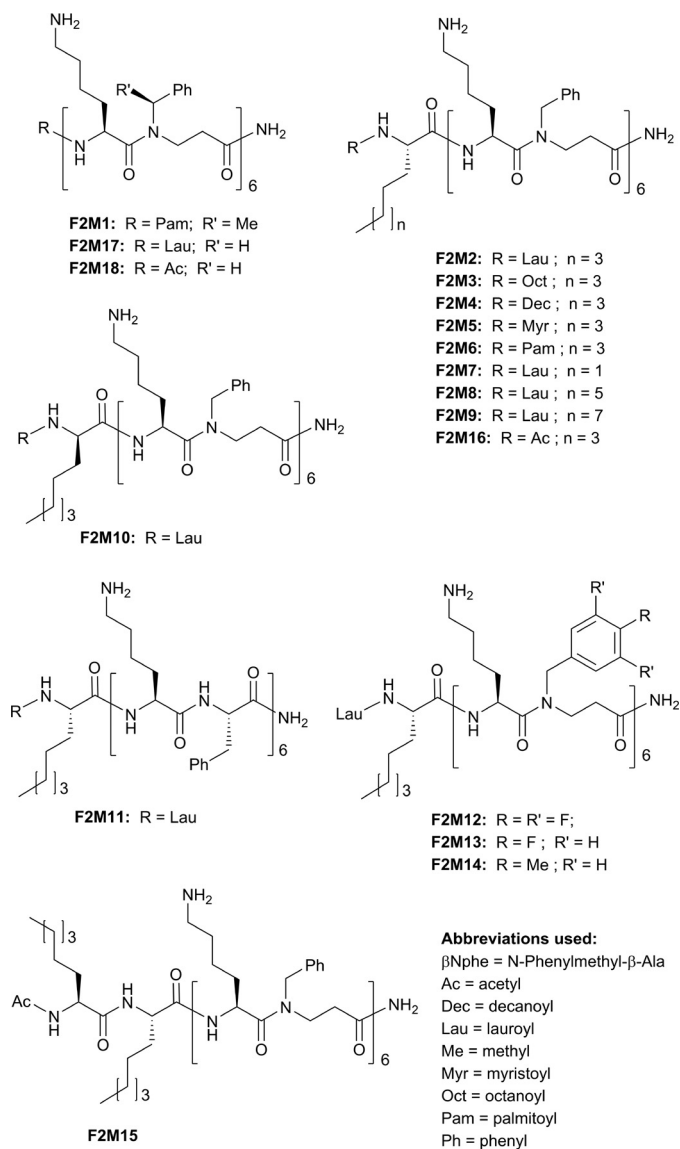Oct = octanoyl
Pam = palmitoyl
Ph = phenyl

FIGURE 2. **Chemical structures of peptidomimetics (F2M1–F2M19) investigated in the study.** Shown is an overview of the structures and nomenclature of the peptidomimetics F2M1–F2M18 used in this study.

tion, selectively through FPR2, with a potency comparable with that of the hitherto most potent FPR2-selective inhibitor known, namely the gelsolin-derived peptide PBP$_{10}$ (RhB-QRLFQVKGRR) functionalized with an N-terminal rhodamine B moiety (23, 24).

In the present work, we have identified a novel FPR2-interacting peptidomimetic, F2M2, (Lau-((*S*)-Aoc)-(Lys-βNphe)$_6$-NH$_2$; Fig. 2) with NADPH oxidase-activating effect, and which also activates mouse neutrophils through Fpr2. Further analysis of a number of structural variants of F2M2 revealed that the *N*-acyl 2-aminooctanoic acid residue and the peptidomimetic backbone of the molecule both were required for the agonistic activity. This novel class of FPR2-activating peptidomimetic ligands may serve as valuable tools for further delineation of the ligand recognition and signaling by FPR2 as well as for exploration of the therapeutic potential of targeting FPR2 in disease.
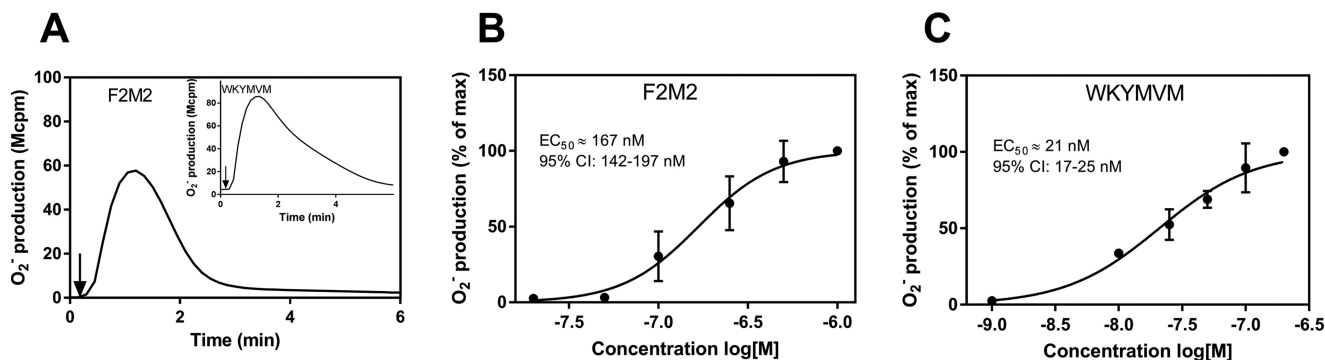
FIGURE 3. **F2M2 activates human neutrophil NADPH oxidase.** Release of superoxide anions was measured by an isoluminol-enhanced chemiluminescence system. Human neutrophils (10^5 cells) were activated by F2M2, and the release of superoxide anions was recorded continuously. *A*, representative response curve of F2M2 (500 nM). The *inset* shows the response induced by the FPR2-specific peptide agonist WKYMVM (100 nM). *Abscissa*, time of study (min); *ordinate*, superoxide production ($10^6 \times$ counts/min (*Mcpm*)). Shown are dose-dependent superoxide release induced by F2M2 (*B*) and WKYMVM (*C*). Data are presented as normalized peak response, and the fitted curve, as well as the EC_{50} value and 95% confidence interval are calculated (mean $\pm$ S.D. (*error bars*), $n > 3$).

Results

The Peptidomimetic F2M2 (Lau-((S)-Aoc)-(Lys-βNphe$_6$-NH$_2$) Activates the NADPH Oxidase in Human Neutrophils—In an earlier study, an array of peptidomimetics representing different structural subclasses was screened, using the neutrophil NADPH oxidase assay as a read-out system (25, 26). A potent FPR2-specific antagonist was identified (22), and in addition, compounds displaying other lipid headgroups were found to partially inhibit the activity induced by known FPR2-specific agonists. This finding was not further explored, but we now show that the peptidomimetic F2M2 (*i.e.* Lau-((*S*)-Aoc)-(Lys-βNphe)$_6$-NH$_2$) in fact activates human neutrophils to produce/release superoxide anions (Fig. 3). The activity induced was concentration-dependent, with an EC_{50} of ~150 nM with a maximal response reached at a concentration of ~500 nM (Fig. 3*B*). Dose response of the FPR2-specific peptide agonist WKYMVM was included for comparison (Fig. 3*C*). The onset and overall kinetics of the F2M2-induced response, reaching the peak in <1 min and subsiding in <5 min (Fig. 3*A*), resembles that induced by other typical GPCR agonists. Based on the structural similarity to the earlier described FPR2 antagonist, F2M2 appeared to represent a novel FPR agonist.

Neutrophil Activation by F2M2 Is Inhibited Only by FPR2-specific Antagonists—To establish the involvement of FPRs in the neutrophil-activating effect of F2M2, two different approaches were used: (i) reciprocal receptor desensitization induced by receptor-specific agonists, and (ii) inhibitory effects of receptor-specific antagonists. Reciprocal receptor desensitization is a process by which a receptor upon stimulation with a specific agonist becomes non-responsive (desensitized) to a second dose, not only of the same agonist but also of other agonists that bind to the receptor involved. Neutrophils triggered by F2M2 were desensitized, not only to a new dose of F2M2 but also to the FPR2-specific peptide agonists PSMα2 and WKYMVM (shown for WKYMVM in Fig. 4*A*). In contrast, F2M2-stimulated human neutrophils were fully responsive or even primed in response to FPR1 agonists (data not shown). The desensitization effect was reciprocal (*i.e.* independent of the order in which the agonists were applied) (Fig. 4*A*). These data suggest that F2M2 activates neutrophils selectively through FPR2 without interference by FPR1.

The receptor preference of F2M2 was investigated also through the use of well-characterized selective FPR antagonists. The two FPR2-specific inhibitors PBP$_{10}$ and F2M1 (22, 23) both completely abolished the response induced by F2M2 (Fig. 4*B*). In contrast, no inhibitory effect was obtained with the FPR1-specific antagonist CsH (27) (Fig. 4*B*). Taken together, these data strongly suggest that F2M2 is a novel FPR2 agonist that triggers an activation of the neutrophil NADPH oxidase in human cells.

The Receptor Specificity of F2M2 Is Confirmed by Using FPR1- and FPR2-overexpressing HL60 Cells—Unequivocal confirmation of the inferred FPR2 specificity of F2M2 was obtained in studies of its ability to activate non-differentiated HL60 cells stably expressing FPR2, measured as an increase in cytosolic Ca^{2+} concentration. F2M2 proved capable of evoking an intracellular Ca^{2+} response in FPR2-expressing cells, with a maximal response at 250 nM and with an EC_{50} value of 50–100 nM (Fig. 5*A*). No response was induced in FPR1-overexpressing HL60 cells (that readily responded to the FPR1-specific agonist fMLF; Fig. 5*B*).

Structural Variants of F2M2 Reveal Substructures of Importance for FPR2 Activation—To gain a deeper insight into the structure-function relationship for FPR2 activation by F2M2, we designed and synthesized an array of variants (Fig. 2 and Table 1). Different structural features were varied independently: (i) the length of the N-terminal fatty acid (*i.e.* F2M3–F2M6); (ii) the length of the linear alkyl side chain in the hydrophobic amino acid (*i.e.* F2M7–F2M9); (iii) the stereochemistry of the 2-aminooctanoic acid (Aoc) (*i.e.* F2M10); (iv) the backbone (α-peptide analogue F2M11); and (v) the hydrophobicity of the repetitive peptidomimetic part (*i.e.* F2M12–F2M14). The neutrophil-activating effects of the structural variants were investigated by their ability to trigger the production of superoxide anions, whereas their preference for FPR2 was evaluated in FPR1- and FPR2-overexpressing HL60 cells.

The fatty acid moiety linked to the N-terminal hydrophobic amino acid proved to be critical for the agonistic effect of F2M2 because F2M15, F2M16, and F2M18 (all lacking the fatty acid) were devoid of activity. The octanoyl analogue (F2M3), displaying an N-terminal fatty acid that was four carbons shorter than F2M2, was likewise devoid of agonistic effect in the tested con-
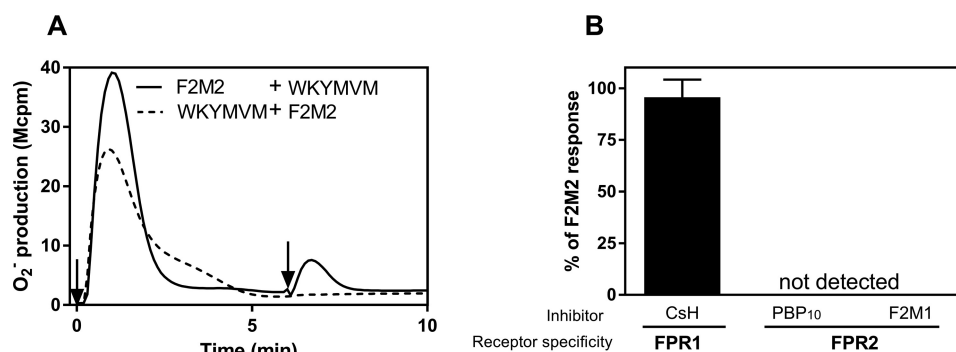
A

B

FIGURE 4. F2M2-induced response in human neutrophils can be desensitized by an FPR2-specific agonist WKYMVM and inhibited by FPR2-specific inhibitors PBP$_{10}$ and F2M1. *A*, primary human neutrophils (10^5 cells) were first stimulated with F2M2 (500 nM; *solid line*) or WKYMVM (50 nM; *dashed line*), and then release of superoxide anions was recorded continuously. After the responses subsided, a second stimulation with WKYMVM (*solid line*) or F2M2 (*dashed line*) was performed on the same batch of cells, as indicated by the *second arrow*. A representative experiment of three independent experiments is shown. *Abscissa*, time of study (min); *ordinate*, superoxide production ($10^6 \times$ counts/min (*Mcpm*)). *B*, effect of FPR inhibitors on the F2M2 response. Human neutrophils (10^5 cells) were preincubated (5 min at 37 °C) with the FPR2-specific inhibitors PBP$_{10}$ (1 μM) or F2M1 (1 μM), or with FPR1-specific inhibitor cyclosporin H (*CsH*) (1 μM) before stimulation with F2M2 (500 nM). Data are presented as percentage of response relative to the control response obtained without inhibitor added (mean ± S.E. (*error bars*), $n > 3$).
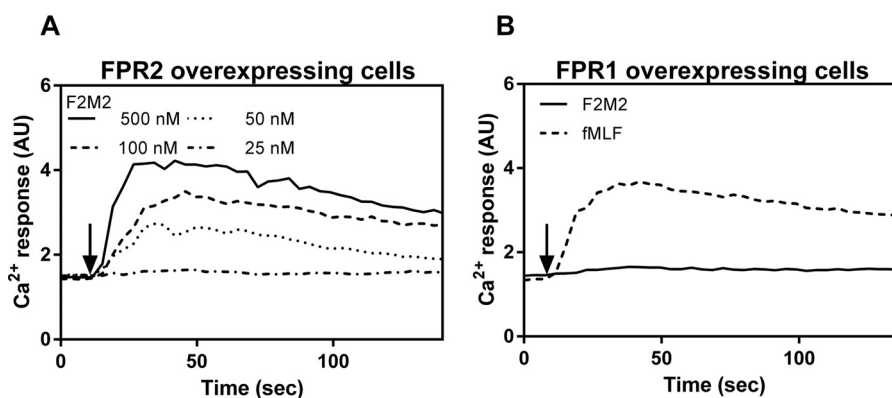
A

B

FIGURE 5. F2M2 triggers calcium response in FPR2- but not in FPR1-overexpressing HL60 cells. The intracellular calcium transient was measured with Fura-2-labeled FPR2- and FPR1-overexpressing HL60 cells. *A*, cells were preincubated (10 min at 37 °C), after which F2M2 at various concentrations (25–500 nM) was added, and then concentration of free cytosolic calcium was monitored by the Fura-2 fluorescence. *B*, F2M2 at up to 250 nM concentrations (shown for 100 nM; *solid line*) did not trigger a calcium response in the HL60 cells overexpressing FPR1, whereas fMLF (positive control, 5 nM; *dashed line*) gave a substantial response. Representative calcium responses (ratio between the fluorescence intensities from excitation at 340 and 380 nm) are shown. *Abscissa*, time of study (s); *ordinate*, fluorescence (arbitrary units (*AU*)).

centration range (up to 2 μM). Decanoyl, myristoyl, and palmitoyl analogues (*i.e.* F2M4–F2M6), displaying alkyl chains that were two carbon atoms shorter or two and four carbon atoms longer than that present in the parent peptidomimetic F2M2, were active but with a reduced potency (Table 1). Shortening of the fatty acid resulted in considerable loss of activity, consistent with the lack of activity of F2M15, being the 2-acetamido-substituted analogue of F2M3.

To test the importance of the hydrophobic amino acid, F2M17, which entirely lacked the Aoc unit, was evaluated for comparison. This lack of an additional alkyl side chain besides the fatty acid resulted in loss of all agonistic activity in the tested concentration range (up to 2 μM), and instead F2M17 acted as an FPR2 antagonist, reducing the response induced by the agonist WKYMVM (Table 1). To examine whether the length of the amino acid alkyl side chain was optimal in F2M2, three analogues were synthesized: F2M7 (displaying (*S*)-norleucine) with a two-carbon shorter side chain, F2M8 (analogue with (*S*)-octylglycine) with two additional carbon atoms in its side chain, and F2M9 (with (*S*)-2-aminododecanoic acid) with a four-carbon longer side chain (Fig. 2). All of these analogues

lacked agonistic activity but maintained an interaction with FPR2, albeit as weak antagonists, underlining the importance of the exact structure of the lipid headgroup to confer agonistic activity. Noticeably, the analogue F2M10 with altered stereochemistry (*i.e.* the Aoc unit in F2M2 was changed from (*S*)-form to (*R*)-form), exhibited significantly reduced neutrophil activation as compared with that of F2M2 (Table 1).

The influence of the peptidomimetic backbone on the agonistic activity of F2M2 was assessed by testing the α-peptide analogue (*i.e.* F2M11). The peptide was entirely inert in the activation assays, indicating that the α-peptide/β-peptoid peptidomimetic backbone is crucial for the ability of this novel agonist type to activate FPR2 (Table 1). Next, we examined the effects of increased hydrophobicity of the peptoid residues via introduction of fluorine or methyl substituents on the benzyl side chain of βNphe (F2M12–F2M14; Fig. 2). These more hydrophobic analogues displayed higher potencies in activating neutrophils as compared to the parental compound F2M2 (Fig. 6*A* and Table 1), suggesting a highly lipophilic binding site. Interestingly, peptidomimetics F2M13 and F2M14 inhibited/desensitized the response induced by

TABLE 1

Structure-activity relationships of lipidated peptidomimetics for stimulation of human FPR2

+++, full agonist; ++, partial agonist; +, weak agonist; −, no activity; ≪, very weak agonist (no EC_{50} value determined); NT, not tested; βNphe, *N*-phenylmethyl-β-alanine; βNFphe, *N*-(4-fluorophenylmethyl)-β-alanine; βNF₃phe, *N*-(3,4,5-trifluorophenylmethyl)-β-alanine; βNMephe, *N*-(4-methylphenylmethyl)-β-alanine; Aoc, 2-aminooctanoic acid; Dec, decanoyl; Dod, 2-aminododecanoic acid; Lau, lauroyl; Lys, ʟ-lysine; Myr, myristoyl; Nle, norleucin; Oct, octanoyl; OGly, octylglycine; Pam, palmitoyl; Phe, ʟ-phenylalanine.

Compound[a]	Structure	NADPH-oxidase response EC_{50} value[b]	Ca²⁺ response in HL60 cells overexpressing	
			FPR2	FPR1
		*n*M		
F2M2	Lau-((*S*)-Aoc)-(Lys-βNphe)₆-NH₂	167 (142–197)	+++	−
Variation of N-terminal fatty acid length				
F2M3	Oct-((*S*)-Aoc)-(Lys-βNphe)₆-NH₂	−	−	−
F2M4	Dec-((*S*)-Aoc)-(Lys-βNphe)₆-NH₂	≪	+	−
F2M5	Myr-((*S*)-Aoc)-(Lys-βNphe)₆-NH₂	176 (153–202)	++	−
F2M6	Pam-((*S*)-Aoc)-(Lys-βNphe)₆-NH₂	173 (151–198)	++	−
Incomplete lipid headgroup				
F2M15	Ac-((*S*)-Aoc-(*S*)-Aoc)-(Lys-βNphe)₆-NH₂	−	−	−
F2M16	Ac-((*S*)-Aoc)-(Lys-βNphe)₆-NH₂	−	NT	−
F2M17	Lau-(Lys-βNphe)₆-NH₂	Antagonist[c]	NT	−
F2M18	Ac-(Lys-βNphe)₆-NH₂	−	NT	−
Variation of hydrophobic amino acid				
F2M7	Lau-((*S*)-Nle)-(Lys-βNphe)₆-NH₂	Antagonist	−	−
F2M8	Lau-((*S*)-OGly)-(Lys-βNphe)₆-NH₂	Antagonist	−	−
F2M9	Lau-((*S*)-Dod)-(Lys-βNphe)₆-NH₂	Antagonist	−	−
Alternative stereochemistry of Aoc				
F2M10	Lau-((*R*)-Aoc)-(Lys-βNphe)₆-NH₂	≪	++	−
Comparison with peptide backbone structure				
F2M11	Lau-((*S*)-Aoc)-(Lys-Phe)₆-NH₂	−	−	−
Variation of β-peptoid hydrophobicity				
F2M12	Lau-((*S*)-Aoc)-(Lys-βNF₃phe)₆-NH₂	−	−	−
F2M13	Lau-((*S*)-Aoc)-(Lys-βNFphe)₆-NH₂	75 (66–85)	+++	−
F2M14	Lau-((*S*)-Aoc)-(Lys-βNMephe)₆-NH₂	89 (78–102)	+++	−

[a] As indicated in Fig. 1.

[b] EC_{50} values are calculated based on data from three independent experiments. 95% confidence intervals (asymmetrical) are shown in parentheses.

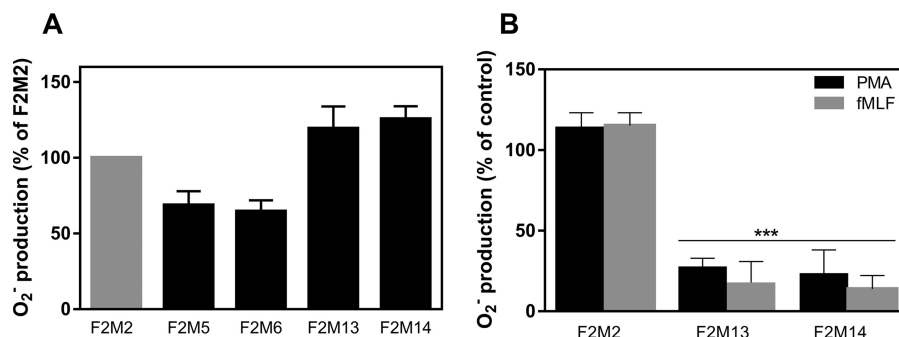[c] Measured as inhibition of the response induced by WKYMVM.

FIGURE 6. Effect of the F2M2 analogues with increased hydrophobicity on the fMLF and PMA response in human neutrophils. *A*, human neutrophils (10⁵ cells) were stimulated with 500 nM F2M2 (*gray bar*) or its analogues (*black bars*), and then release of superoxide anions was recorded continuously. Data are presented as percentage of the peak response obtained in the absence of peptidomimetics. *B*, human neutrophils (10⁵ cells) were preincubated with or without peptidomimetics (500 nM) for 5 min at 37 °C before stimulation with PMA (50 nM; *black bars*) or fMLF (100 nM; *gray bars*), and then release of superoxide anions was measured as chemiluminescence. Data are presented as percentage of the peak response obtained in the absence of peptidomimetics. One-way analysis of variance with F2M2 as control was used for statistical analysis. ***, $p \leq 0.001$. (mean ± S.D. (*error bars*), $n = 3$).

FPR2 agonists but also by the FPR1 agonist fMLF as well as by phorbol 12-myristate 13-acetate (PMA), a PKC activator that bypasses membrane receptors (Fig. 6*B*), indicating that increased hydrophobicity leads to reduced FPR2 selectivity. In addition, increasing hydrophobicity appeared to confer cytotoxic properties to the heavily fluorinated analogue F2M12, which activated neutrophils to produce superoxide at low concentrations (≤250 nM) but rendered the cells non-responsive at higher concentrations (up to 1 μM) and abrogated the responsiveness to a second stimulation with PMA (data not shown).

All agonistic variants of F2M2, described above, displayed FPR2 selectivity, because the responses induced in human neutrophils were inhibited by the FPR2-specific inhibitor PBP₁₀ but were unaffected by the FPR1-specific inhibitor CsH (data not shown). In addition, all peptidomimetic agonist variants
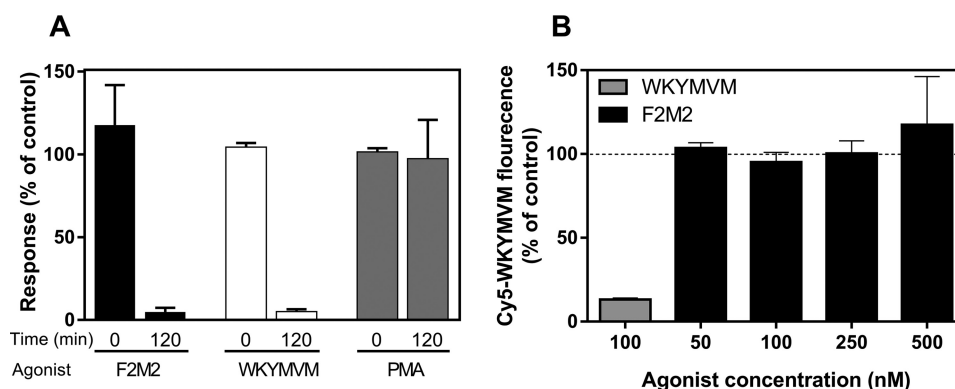
FIGURE 7. **F2M2 triggers a pertussis toxin-sensitive response and does not compete with WKYMVM for binding to FPR2 on human neutrophils.** *A*, neutrophils (10^6 cells/ml) were incubated with or without pertussis toxin (500 ng/ml, 37 °C, time 0 and 120 min) before stimulation with F2M2 (500 nM) and WKYMVM (100 nM). The PMA (50 nM) response was included as a negative control. Superoxide production was measured continuously. Data are expressed as percentage of the peak response from respective controls (pertussis toxin-non-treated), mean ± S.D. (*error bars*), $n = 3$. *B*, neutrophils (10^6 cells/ml) were incubated with different concentrations of F2M2 as indicated (*black bars*) on ice for 10 min, after which the fluorescently labeled FPR2 agonist Cy5-WKYMVM (1 nM) was added, and incubation was continued for 60 min. Cells pretreated with unlabeled WKYMVM (100 nM; *gray bar*) or without agonist (total binding) before the addition of Cy5-WKYMVM (1 nM) were included. Data are expressed as a percentage of total binding (mean fluorescence intensity ± S.E. (*error bars*), $n = 3$).

triggered calcium transients in FPR2-overexpressing cells, but not in FPR1-overexpressing cells (Table 1).

Taken together, F2M2 was the most potent FPR2-selective neutrophil-activating peptidomimetic of the variants tested. In accordance with this, the interaction of F2M2 with primary human neutrophils was investigated further to shed more light on how FPR2 regulation is mediated by this novel type of agonist.

F2M2 Triggers a Pertussis Toxin-sensitive Response and Does Not Compete with WKYMVM for Binding to FPR2 on Human Neutrophils—FPRs are known to couple to pertussis toxin-sensitive G_i-protein for signaling. To characterize the effect of pertussis toxin on the F2M2 response, neutrophils were treated with pertussis toxin at different time points before stimulation with F2M2. Also, the FPR2 agonist WKYMVM and the receptor-independent PMA were included as positive and negative controls, respectively, at each time point. Pertussis toxin treatment for 2 h abolished both the WKYMVM- and F2M2-induced superoxide production (Fig. 7*A*), showing that a pertussis toxin-sensitive G-protein is involved in signaling downstream from FPR2 upon F2M2 binding. The cell response to PMA was unaffected by pertussis toxin (Fig. 7*A*).

To study the binding mode of F2M2 on FPR2, we quantified the binding of Cy5-WKYMVM to neutrophils in the presence or absence of F2M2 by flow cytometry. Binding of Cy5-WKYMVM was inhibited by excess (100-fold) non-labeled WKYMVM (Fig. 7*B*) but not by excess (500-fold) F2M2 (Fig. 7*B*). These data suggest that F2M2 and WKYMVM most likely interact with different binding sites.

Priming of Superoxide Release from Human Neutrophils Triggered by F2M2—Neutrophil production/release of superoxide anions in response to other FPR agonist differs in magnitude, depending on the state of the cells, being either naïve (low responders) or primed (high responders). Priming of neutrophils can be achieved with a non-activating cytokine, such as TNF-α. Disruption of the neutrophil cytoskeleton with an inhibitor of actin polymerization, such as latrunculin A, also primes the response to FPR agonists (9, 28, 29). In accordance

with this, neutrophils pretreated with TNF-α at 37 °C for 20 min produced increased amounts of superoxide in response to F2M2 when compared with the response induced in naïve cells (2-fold increase). For neutrophils pretreated with latrunculin A, the F2M2 response was not only augmented but was also prolonged as compared with that observed in naïve cells (Fig. 8*A*), with an EC_{50} ~110 nM (Fig. 8*B*).

FPR2 Desensitized with F2M2 Can Be Reactivated through Receptor Cross-talk—We have earlier shown that agonist-occupied and -desensitized FPRs can be reactivated to produce superoxide when the cytoskeleton is disrupted by latrunculin A (Lat A) (30). The molecular basis for this reactivation is an inhibition of the coupling of ligand-receptor complexes to the actin cytoskeleton, an interaction that normally terminates the response and desensitizes the receptors (30, 31). Recently, we described another, more physiological mode of FPR1 and FPR2 reactivation, involving receptor cross-talk with $P2Y_2R$ and PAFRs upon stimulation with the receptor-specific agonists ATP and PAF, respectively (32, 33). The signals involved in this type of receptor cross-talk have not yet been identified.

Here, we characterized neutrophil desensitization and reactivation when F2M2 was used as the desensitizing agonist. Similar to neutrophils desensitized with the FPR2 agonist WKYMVM, cells desensitized with F2M2 could be reactivated to produce superoxide upon disruption of the cytoskeleton with Lat A (Fig. 9*A*). In addition, neutrophils could be reactivated through the described receptor cross-talk mechanism when F2M2-desensitized cells subsequently were stimulated with PAF (Fig. 9*B*). Thus, the PAF-induced response in F2M2-desensitized cells was primed up to 4-fold as compared to the PAF response in naïve cells (Fig. 9*B*). Interestingly, the PAF response was sensitive not only to a PAFR-specific inhibitor (data not shown), but also to PBP_{10}, a potent FPR2 inhibitor (Fig. 9*B*). This is in accordance with a PAF-induced reactivation of FPR2 in the WKYMVM-desensitized cells (32). The F2M2-desensitized neutrophils were reactivated also when PAF was replaced by the $P2Y_2R$ agonist ATP (data not shown), a recep-
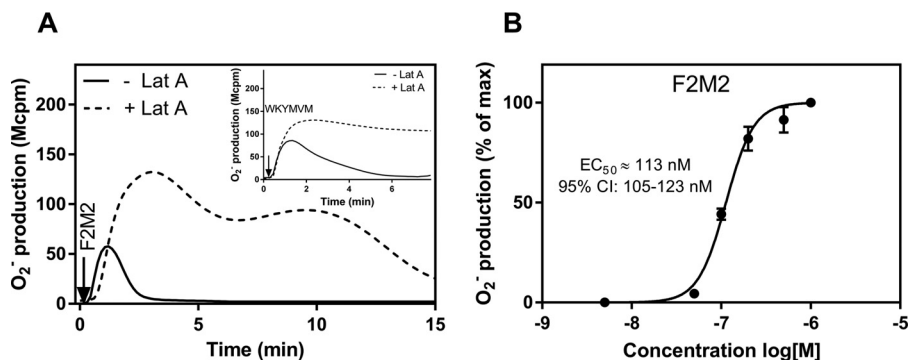
A

B

FIGURE 8. **F2M2-induced superoxide release in human neutrophils can be primed by Lat A.** Naive human neutrophils (10^5 cells) were pretreated with Lat A (25 ng/ml) for 5 min at 37 °C, after which the cells were activated by different concentrations of F2M2 (1 μM, 500 nM, 250 nM, 100 nM, and 50 nM), and then release of superoxide anions was recorded continuously. *A*, representative response curves for F2M2 (500 nM) in naive cells (*solid line*) and in cells primed with Lat A (*dotted line*) are shown. For comparison, the corresponding WKYMVM response is shown in the *inset*. *Abscissa*, time of study (min); *ordinate*, superoxide production ($10^6 \times$ counts/min (*Mcpm*)). *B*, data for F2M2 are presented as normalized peak response with the fitted curve. EC_{50} value and 95% confidence interval are calculated from three independent experiments (mean $\pm$ S.D. (*error bars*)).
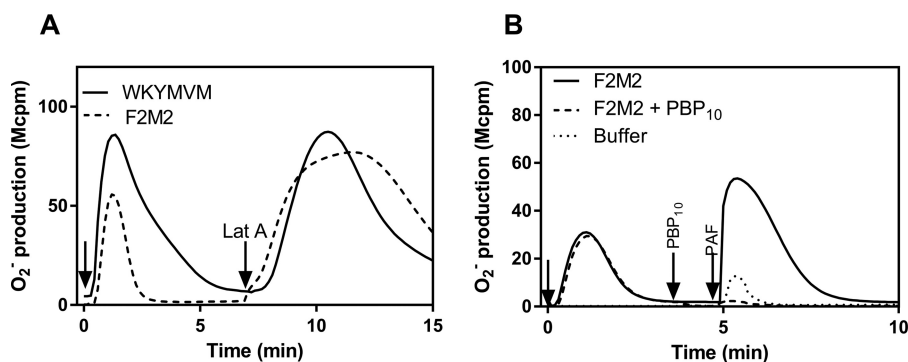
A

B

FIGURE 9. **F2M2-desensitized FPR2 can be reactivated to release superoxide upon stimulation with Lat A or PAF in human neutrophils.** *A*, naive neutrophils (10^5 cells) were desensitized with WKYMVM (100 nM; *solid line*) or F2M2 (500 nM; *dashed line*), and subsequently FPR2 reactivation was achieved with the actin cytoskeleton-disrupting agent Lat A. A representative experiment is shown, and the time point for the addition of agonists is indicated by the *first arrow*, whereas the time point for Lat A stimulation (10 ng/ml) is indicated by the *second arrow*. *Abscissa*, time of study (min); *ordinate*, superoxide production ($10^6 \times$ counts/min (*Mcpm*)). *B*, naive neutrophils (10^5 cells) were first desensitized with F2M2 (500 nM) and subsequently stimulated with PAF as indicated by the *arrow* (100 nM; *solid line*), and release of superoxide anions was recorded continuously. When required, the FPR2-specific inhibitor PBP$_{10}$ (1 μM) was added 1 min before PAF stimulation (*dashed line*). The PAF responses induced in naive cells (*dotted line*) are shown for comparison. *Abscissa*, time of study (min); *ordinate*, superoxide production ($10^6 \times$ counts/min).

tor-ligand pair earlier shown to display cross-talk with desensitized FPR2 (34).

In summary, the novel FPR2-activating F2M2 triggers very similar signaling pathways as the conventional FPR2 agonist WKYMVM, because both switch FPR2 into a desensitized state that can be reactivated via disruption of the cytoskeleton as well as by signals generated by other GPCRs when activated by their respective specific agonists.

F2M2 Activates Mouse Neutrophils and the Targeted Receptor Is Fpr2—The profile of ligand recognition differs considerably between mouse and human FPRs, and very few ligands have been shown to possess comparable agonistic potency on both human and mouse receptors. This is illustrated by the fact that the hexapeptide WKYMVM is a potent full agonist for FPR2 in human neutrophils ($EC_{50} \sim$ 40 nM) (35), whereas it is a weaker agonist (EC_{50} value >200 nM) in mouse neutrophils because it only activates Fpr2 partially (10B). Having identified F2M2 as a novel FPR2 agonist, we examined its ability to trigger superoxide production in mouse neutrophils. Interestingly, F2M2 activated mouse neutrophils ($EC_{50} \sim$ 80 nM; Fig. 10A) with higher efficacy than WKYMVM but with very similar kinetics (Fig. 10B).

When Fpr2$^{-/-}$ neutrophils were exposed to F2M2, we found the cells to be non-responsive to F2M2 (Fig. 10B), demonstrating that Fpr2 is the receptor involved in the F2M2-mediated response in mouse cells.

The neutrophil-activating effects of the structural analogues of F2M2 were also determined in mouse neutrophils, and most of the analogues that were able to activate human neutrophils also activated mouse neutrophils (Table 2). In addition, all agonistic analogues failed to activate Fpr2$^{-/-}$ neutrophils (Table 2), demonstrating that Fpr2 selectivity was retained also for the F2M2 structural analogues. Overall, the structure-function relationships for the lipidated peptidomimetics in mouse Fpr2 activation (Table 2) were similar to those found for the human FPR2 (Table 1). The presence of the entire N-terminal lipid headgroup proved equally important for activation of mouse neutrophils, as demonstrated by the lack of agonistic activity of the analogues lacking either the fatty acid (*i.e.* F2M16), the hydrophobic amino acid (F2M17), or the entire headgroup (F2M18). As for FPR2, the length of the N-terminal fatty acid was critical for potency on Fpr2, as demonstrated by the decreased (or even abolished) activity of peptidomimetics F2M3, F2M4, and F2M16.
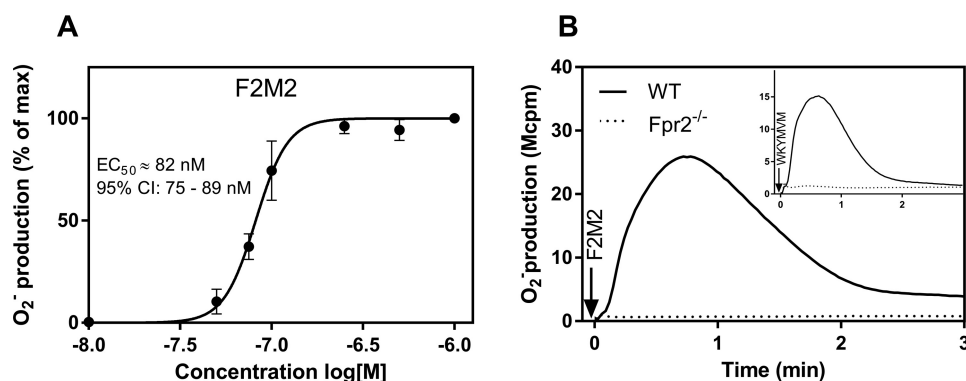
FIGURE 10. **Effect of F2M2 in activation of mouse neutrophils derived from wild-type and Fpr2-deficient mice.** Mouse neutrophils were isolated from bone marrow of wild-type C57/B6 mice and mice deficient in Fpr2 (Fpr2$^{-/-}$). Cells (10^5 cells) were primed with TNF-α for 20 min at 37 °C before stimulation with agonists. *A*, dose-dependent release of superoxide induced by F2M2 was recorded, and data are presented as normalized peak response with the fitted curve. EC$_{50}$ value and 95% confidence interval are calculated from three independent experiments (mean $\pm$ S.D. (*error bars*)). *B*, representative superoxide production induced by F2M2 (500 nM) in wild-type (*solid line*) and Fpr2$^{-/-}$ (*dashed line*) neutrophils. *Inset*, a representative WKYMVM (200 nM) response for comparison. *Abscissa*, time of study (min); *ordinate*, superoxide production ($10^6 \times$ counts/min (*Mcpm*)).

TABLE 2

Structure-activity relationships of lipidated peptidomimetics for stimulation of mouse Fpr2

$-$, no activity; NT, not tested; $\ll$, a very weak agonist (no EC$_{50}$ value determined); βNphe, *N*-phenylmethyl-β-alanine; βNFphe, *N*-(4-fluorophenylmethyl)-β-alanine; βNF$_3$phe, *N*-(3,4,5-trifluorophenylmethyl)-β-alanine; βNMephe, *N*-(4-methylphenylmethyl)-β-alanine; Aoc, 2-aminooctanoic acid; Dec, decanoyl; Dod, 2-aminodode-canoic acid; Lau, lauroyl; Lys, L-lysine; Myr, myristoyl; Nle, norleucin; Oct, octanoyl; OGly, octylglycine; Pam, palmitoyl; Phe, L-phenylalanine.

Compound[a]	Structure	NADPH-oxidase response EC$_{50}$ value[b]	
		WT	Fpr2$^{-/-}$
		*n*M	
F2M2	Lau-((*S*)-Aoc)-(Lys-βNphe)$_6$-NH$_2$	82 (75–89)	$-$
Variation of N-terminal fatty acid length			
F2M3	Oct-((*S*)-Aoc)-(Lys-βNphe)$_6$-NH$_2$	$-$	$-$
F2M4	Dec-((*S*)-Aoc)-(Lys-βNphe)$_6$-NH$_2$	185 (165–209)	$-$
F2M5	Myr-((*S*)-Aoc)-(Lys-βNphe)$_6$-NH$_2$	123 (108–140)	$-$
F2M6	Pam-((*S*)-Aoc)-(Lys-βNphe)$_6$-NH$_2$	139 (120–161)[c]	$-$
Incomplete lipid headgroup			
F2M15	Ac-((*S*)-Aoc-(S)-Aoc)-(Lys-βNphe)$_6$-NH$_2$	$-$	NT
F2M16	Ac-((*S*)-Aoc)-(Lys-βNphe)$_6$-NH$_2$	$-$	NT
F2M17	Lau-(Lys-βNphe)$_6$-NH$_2$	$-$	NT
F2M18	Ac-(Lys-βNphe)$_6$-NH$_2$	$-$	NT
Variation of hydrophobic amino acid			
F2M7	Lau-((*S*)-Nle)-(Lys-βNphe)$_6$-NH$_2$	$-$	$-$
F2M8	Lau-((*S*)-OGly)-(Lys-βNphe)$_6$-NH$_2$	$-$	$-$
F2M9	Lau-((*S*)-Dod)-(Lys-βNphe)$_6$-NH$_2$	Antagonist[d]	$-$
Alternative stereochemistry of Aoc			
F2M10	Lau-((*R*)-Aoc)-(Lys-βNphe)$_6$-NH$_2$	$\ll$	$-$
Comparison with peptide backbone structure			
F2M11	Lau-((*S*)-Aoc)-(Lys-Phe)$_6$-NH$_2$	$-$	$-$
Variation of β-peptoid hydrophobicity			
F2M12	Lau-((*S*)-Aoc)-(Lys-βNF$_3$phe)$_6$-NH$_2$	$-$	$-$
F2M13	Lau-((*S*)-Aoc)-(Lys-βNFphe)$_6$-NH$_2$	$\ll$	$-$
F2M14	Lau-((*S*)-Aoc)-(Lys-βNMephe)$_6$-NH$_2$	$\ll$	$-$

[a] As indicated in Fig. 1.
[b] EC$_{50}$ values are calculated based on data from three independent experiments. 95% confidence intervals (asymmetrical) are shown in parentheses.
[c] Symmetrical confidence interval is presented for this compound due to the very steep Hill slope curve.
[d] Measured as inhibition of the response induced by WKYMVM.

Likewise, the nature of the hydrophobic amino acid displaying a linear alkyl side chain proved to be essential for the Fpr2-activating capacity of F2M2, because only the analogue displaying the Aoc residue exhibited agonistic activity. However, the structure-function relationships of the analogues displaying hydrophobic amino acids with different side chain lengths (F2M7–F2M9) were different for mouse and human neutrophils, because only analogue F2M9 (with a side chain four car-bon atoms longer than Aoc in F2M2) showed antagonistic activity in mouse neutrophils, whereas the other analogues were inert at concentrations up to 1 μM (Table 2).

Discussion

During the last two decades, numerous molecules with mod-ulatory effects on FPRs (in particular FPR2) have been identi-fied (2, 3). It is obvious from these studies that FPR2 is a pro-

miscuous receptor recognizing molecules ranging from proteins and peptides to pepducins and small molecules via a not yet defined binding pocket. Recently, we identified a potent FPR2-specific antagonist (F2M1) from a small library of proteolytically stable lipidated α-peptide/β-peptoids (22), and we now show that this class of compounds also comprises FPR2-specific agonists with F2M2 as a potent lead compound.

In the present study, we have characterized a peptidomimetic (F2M2, Lau-((S)-Aoc)-(Lys-βNphe)$_6$-NH$_2$), having an α-peptide/β-peptoid backbone displaying an N-terminal Aoc residue conjugated with a 12-carbon fatty acid chain, as a novel FPR2/Fpr2 agonist. Upon interaction with its receptor, F2M2 potently triggers superoxide release from both human and mouse neutrophils. In addition, several analogues of F2M2 also proved to be FPR2/Fpr2-selective agonists. Structure-activity relationships for F2M1 (FPR2 antagonist) and F2M2 (FPR2 agonist) demonstrate an essential role of the fatty acid in mediating the interaction of these peptidomimetics with FPR2/Fpr2 (this study and Ref. 22). Although the fatty acid plays a key role in FPR2 modulation, it is not simply a molecular pattern recognized by FPR2 because many ligands lacking a fatty acid moiety bind to FPR2, and many lipidated peptides/peptidomimetics are devoid of activity. Similarly, pepducins also contain a fatty acid, but in contrast, they consist of natural amino acid sequences derived from one of the intracellular loops of GPCRs (36). Both activating and inhibitory pepducins have been shown to target FPR2, and these include pepducins with amino acid sequences originating from FPR2, FPR1, CXCR4, and the ATP receptor P2Y$_2$R (2, 37–39). The precise mechanism of action of pepducins is not known, but the fact that both lipidated peptidomimetics and pepducins interact with FPR2 indicates a not yet understood role of FPR2 in recognition of lipopeptides and lipidated peptidomimetics. It has been proposed that when it comes to the action of a pepducin, the fatty acid anchors the molecule in the cell membrane, facilitating interaction between the peptide part and the receptor at the signaling interface (40). However, comparison of the structure-activity relationships for analogues of the FPR2-activating peptidomimetic F2M2 with those of the FPR2-inhibiting F2M1 described earlier (22) reveals differences in the optimal length of the N-terminal fatty acid for activation (12 carbons) and inhibition (at least 16 carbons). If the N-terminal fatty acid in these FPR2 ligands merely anchors the compounds in the lipid bilayer, it would be expected that the optimal length would be similar for agonists and antagonists; however, the observation that longer fatty acids (palmitoyl and myristoyl) are less favorable than the less membrane-interacting Lau moiety rather suggests an affinity-related role for the N-terminal fatty acid. Nevertheless, the mechanisms involved in FPR2 recognition of these lipidated molecules are not known. In addition to the N-terminal fatty acid, F2M2 also contains a hydrophobic amino acid displaying a six-carbon alkyl side chain (*i.e.* Aoc), and this side chain was found to be equally important for the agonistic activity. The finding that relatively small changes (2 carbon atoms) in the length of the amino acid alkyl chain in fact resulted in a dissimilar activity profile suggests that the receptor binding pocket for F2M2 is quite restricted.

We show that F2M2-activated neutrophils are desensitized to a new dose of FPR2-specific agonist but are fully responsive to an FPR1 agonist. Furthermore, the desensitized FPR2 can be reactivated by agents that disrupt the actin cytoskeleton, an important player in FPR desensitization. β-arrestin-mediated signaling has been shown to be important for desensitization and endocytosis of many ligand-occupied GPCRs and for downstream activation of ERK1/2, but the role of arrestin in signaling and functional responses for the FPRs has not been clearly defined. Based on results obtained using a simple and straightforward system in transfected cells to measure β-arrestin translocation, both FPR1 and FPR2 can be concluded to trigger a translocation of β-arrestin (23). However, it has been shown that the endocytic process, resulting in internalization of agonist-occupied FPR1 and blocking of G-protein interaction with phosphorylated FPR1, occurs independently of β-arrestin. In addition, both FPR1- and FPR2-induced ERK1/2 activation (downstream from β-arrestin for many GPCRs) depends on Gα$_i$ signaling and is independent of β-arrestin (41, 42). Internalization of FPR2, but not ERK1/2 activation, is found to be compromised in cells lacking arrestins, and it has also been shown that, although the basic FPR1 signaling occurs independently of arrestins, this signaling route is important for the receptor recycling process (42). None of the previous studies on the link between FPRs and arrestins have been performed in primary neutrophils, suggesting that further studies are needed to determine the importance of the β-arrestin route in signaling by the FPRs in naive neutrophils. GPCR desensitization typically accompanies receptor internalization from the surface; however, determination of surface levels of FPRs in neutrophils is technically difficult due to the fact that these cells have large pools of FPRs stored in subcellular granules that are mobilized to the surface upon activation (43, 44).

The precise role of FPR2/Fpr2 in a complex *in vivo* biological system is not known. At the signaling level, both pro- and anti-inflammatory signal transduction patterns have been reported for FPR2. In addition, complex ligand recognition profiles across species have been noticed. With respect to the latter, a prominent example is the prototype FPR1 agonist fMLF, which is a potent agonist for the human receptor but a poor agonist for the mouse ortholog (for a recent review, see Ref. 2). In contrast to the large number of identified ligands with well characterized pharmacology with respect to human FPRs, little is known for the mouse receptor counterparts. One reason for this is the different expansion history of the FPR family across species (e.g. there are three members in the human genome in contrast to at least eight members in mouse) (45). In fact, most of the investigations in mouse models aiming to understand the role of FPRs involve ligands optimized for human FPRs (13). To our knowledge, most of the identified ligands for FPRs are not active or lack receptor specificity in mouse cells. Our data reveal that F2M2 also possesses agonist activity in mouse neutrophils, and compared with the most potent FPR2-activating hexapeptide, WKYMVM (35), F2M2 is a more potent Fpr2 agonist (EC$_{50}$ value of 80 nM as compared with >200 nM for WKYMVM). Although the potencies of F2M2 for the human (157 nM) and mouse (80 nM) receptors are somewhat different, the structure-activity relationships for its analogues are similar

in mice and humans. A minor difference is a more pronounced dependence on the length of the amino acid alkyl side chain in mouse neutrophils for analogues possessing antagonistic activity. On the other hand, a shorter C_{10} fatty acid could be accommodated in the murine receptor. The similarity in structure-activity relationships across species suggests that the mechanisms of the ligand-receptor interactions are similar and that common structural features facilitate receptor activation in both human and mouse systems. Clearly, more *in vitro* studies aiming at elucidating the prerequisites for ligand recognition and signaling mechanism of this receptor both in humans and in mice are required. The knowledge obtained should improve our understanding of the role of this receptor in a complex biological environment.

The identification of F2M2 in the present study as a selective cross-species agonist for both FPR2 and Fpr2 has significant potential for further advancing the exploration of FPR2 pharmacology. In conclusion, a novel class of proteolytically stable agonists for FPR2/Fpr2 that activate neutrophils has been identified. These peptidomimetics should serve as valuable tools for further investigation of receptor pharmacology and mechanistic studies of FPR2-mediated immunomodulation in both mice and humans.

Experimental Procedures

Ethics Statement for Human Blood and Mice—Buffy coat samples were obtained from the blood bank at Sahlgrenska University Hospital. Ethics approval was not needed because the buffy coats were provided anonymously and could not be traced back to a specific individual. This is in line with Swedish legislation section code 4§ 3p SFS 2003:460 (Lag om etikprövning av forskning som avser människor).

Wild-type C57BL/6 mice (6–8 weeks of age) obtained from Charles River Laboratories and Fpr-rs2-deficient (Fpr2$^{-/-}$) mice, generated as described previously (11), were housed in the animal facility of the Department of Rheumatology and Inflammation Research, University of Gothenburg. Mice were kept under standard temperature and light conditions and fed laboratory chow and water *ad libitum*. The animal study was approved by the ethics committee for animal research in Gothenburg.

Chemicals, Reagents, and Peptides—HRP, PMA, and pertussis toxin were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). PBP_{10} peptide was obtained from Caslo Laboratory (Lyngby, Denmark), whereas cyclosporin H was kindly provided by Novartis Pharma (Basel, Switzerland). The hexapeptide WKYMVM was purchased from AltaBioscience (University of Birmingham, Birmingham, UK). TNF-α, the formylated peptide fMLF, PMA, and Lat A were purchased from Sigma-Aldrich. PAF was from Avanti Polar Lipids Inc. (Alabaster, AL). All peptides were dissolved in DMSO to a concentration of 10 mM and stored at −80 °C until use. Further dilutions were made in Krebs-Ringer phosphate buffer that was supplemented with glucose (10 mM), Ca^{2+} (1 mM), and Mg^{2+} (1.5 mM) (KRG; pH 7.3). RPMI 1640, FCS, penicillin and streptomycin, and G418 were from PAA Laboratories GmbH. Hanks' balanced salt solution was from Gibco, and Percoll was from GE Healthcare. Fura-2 was purchased from Life Technologies, Inc.

The Cy5-WKYMVM peptide was from Phoenix Pharmaceuticals (Burlingame, CA).

Synthesis and Characterization of Peptidomimetics— Dimeric α-peptide/β-peptoid building blocks were prepared by using a previously published protocol (46) with modifications (see [supplemental material](#) for details). Peptidomimetics F2M2–F2M10 and F2M12–F2M18 were prepared by using previously reported procedures (21) with modifications (see [supplemental material](#) for details). Peptide F2M11 was prepared by microwave-assisted Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)-based solid-phase peptide synthesis (on a Rink Amide AM resin) on a CEM Liberty microwave peptide synthesizer (see [supplemental material](#) for details). The peptidomimetics were dissolved in PBS before use in cell assays.

Isolation of Human and Mouse Neutrophils—Human peripheral blood polymorphonuclear neutrophils were isolated from buffy coats (Blood Center, Sahlgrenska University Hospital, Gothenburg, Sweden) obtained from apparently healthy adults (47). After storage overnight at ambient temperature, erythrocytes were depleted by dextran sedimentation at $1 \times g$, and the leukocyte mixture was centrifuged on a Ficoll-Paque gradient. After a hypotonic lysis of the remaining erythrocytes, the polymorphonuclear neutrophils were washed twice, resuspended (1×10^7 cells/ml) in KRG, and kept on melting ice until use.

Mouse bone marrow-derived neutrophils from 8–10 weeks old wild-type and Fpr2-deficient C57/B6 mice were isolated as described earlier (9, 48). Briefly, bone marrow cell suspension was collected by flushing the femurs and tibias with 10 ml of cold KRG containing 10 mM glucose and 1.5 mM Mg^{2+}, pH 7.3. A three-layer Percoll density gradient composed of 1.095, 1.085, and 1.070 g/ml was used to enrich neutrophils from the total leukocyte population. Neutrophils were collected from the 1.085/1.095 g/ml interface after centrifugation at $500 \times g$ for 30 min at 4 °C. The contaminating erythrocytes were hypotonically lysed, and the remaining neutrophils were washed and resuspended in KRG and stored on ice until use.

Expression of FPRs in Undifferentiated HL60 Cells—The stable expression of FPR1 and FPR2 in undifferentiated HL60 cells has been described previously (23, 49). To prevent autodifferentiation, cells were passaged twice a week before reaching a density of 2×10^6 cells/ml. At each passage, an aliquot of cells was centrifuged, the supernatant was discarded, and the cell pellet was resuspended in fresh culture medium containing RPMI 1640 supplemented with FCS (10%), penicillin/streptomycin (1%), L-glutamine (2 mM), and the selection antibiotic G418 (1 mg/ml). Cells are routinely checked for functionality and selective expression of FPR1 or FPR2, respectively, using the FPR1-specific fMLF and FPR2-specific WKYMVM ligand. The FPR1 cells respond to fMLF but not WKYMVM, whereas the FPR2 cells respond to WKYMVM but not to fMLF.

Neutrophil NADPH Oxidase Activity—Superoxide anion production was determined by using the isoluminol-enhanced chemiluminescence system (25, 26). The NADPH oxidase activity was measured in a 6-channel Biolumat LB 9505 luminometer (Berthold Co., Wildbad, Germany) using disposable 4-ml polypropylene tubes with a 1-ml reaction mixture. The release of reactive oxygen species was measured with neutrophils (10^5 cells) in KRG mixed with isoluminol (2×10^{-5} M) and

HRP (4 units/ml). TNF-α (50 ng/ml; 37 °C for 20 min)-primed bone marrow-derived mouse neutrophils were used due to the low level of superoxide release from resting cells. The cells were prewarmed for 5 min at 37 °C in the presence or absence of receptor inhibitors, after which the stimulus was added, and the light emission was recorded continuously.

Changes in the Intracellular Concentration of Ca^{2+}—Undifferentiated stably transfected HL60 cells (2×10^7 cells/ml) were labeled with Fura-2/AM (Molecular Probes, Inc., Eugene, OR), and the change in the cytosolic Ca^{2+} concentration was followed by the use of a PerkinElmer Life Sciences fluorescence spectrophotometer (LC50) as described previously (50, 51). The transient rise in intracellular calcium is presented as the ratio between fluorescence intensities of the emitted light at 510 nm when excited at 340 and 380 nm.

Competitive Receptor Binding—The peptidomimetic F2M2 (1–500 nM) was added to neutrophils (1×10^6 cells/ml) in KRG and incubated on melting ice for 10 min, after which the fluorescently labeled FPR2 agonist Cy5-WKYMVM was added (1 nM), and incubation was continued for 1 h. Samples with Cy5-WKYMVM in the presence or absence of cold WKYMVM (100 nM) were used as controls. The samples were analyzed by flow cytometry using an Accuri flow cytometer.

Data Collection and Statistical Analysis—Data analysis was performed using GraphPad Prism version 7.0. One-way analysis of variance was used for statistical analysis.

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