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A neutrophil inhibitory pepducin derived from FPR1 expected to target FPR1 signaling hijacks the closely related FPR2 instead

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ABSTRACT

Pepducins constitute a unique class of G-protein coupled receptor (GPCR) modulating lipopeptides. Pepducins with inhibitory effects on neutrophils could potentially be developed into anti-inflammatory pharmaceuticals. A pepducin with a peptide sequence identical to the third intracellular loop of FPR1 was found to inhibit neutrophil functions including granule mobilization and superoxide production. This FPR1-derived pepducin selectively inhibited signaling and cellular responses through FPR2, but not FPR1 as expected. Binding to the neutrophil surface of a conventional FPR2 agonist is also inhibited. The fatty acid is essential for inhibition and pepducins with shorter peptides lose in potency. In summary, a pepducin designed to target FPR1 was found to hijack FPR2 and potently inhibit neutrophil functions.

1. Introduction

The inflammatory response constitute an important part of our innate immunity system, but unbalanced or prolonged, inflammation can be detrimental to the host [1]. Immunomodulation has been proposed as an anti-inflammatory therapeutic approach and as neutrophils are important effector cells and the first cells to be recruited to inflammatory sites, these cells have become an attractive target for drug development [2–4]. Recruitment and activation of neutrophils occur through integration of signals from cell-surface G-protein coupled receptors (GPCRs) recognizing endogenous or microbial derived chemoattractants such as N-formylated peptides recognized by formyl peptide receptors (FPRs) [5,6]. Pepducins, lipopeptides composed of a fatty acid conjugated with a short peptide, activate or inhibit signaling of GPCRs [7,8]. The peptide in a given pepducin has an amino acid sequence identical to one of the intracellular domains of the receptor aimed to be targeted, and this is the basis for the suggestion that pepducins interact directly with a cytosolic receptor domain and thereby activate or inhibit the functions of the targeted receptor [7,9]. Many receptor-specific pepducins have been identified, and their therapeutical potentials have been explored with promising results [7,10,11] and they represent a novel allosteric approach to modulation of GPCR signaling. Activating pepducins highly specific for formyl peptide receptor 2 (FPR2) were recently described [12,13]. The two FPRs expressed in human neutrophils (FPR1 and FPR2) share a large sequence similarity (69% amino acid identity) [5,6], but the FPR2 pepducins showed no cross activation of FPR1 [13–15]. The two receptors utilize very similar signaling pathways [16], but the recent disclosure of some fundamental differences [13,17] suggests that the two neutrophil FPRs are not redundant and they may have different roles in inflammation and/or innate immunity.

In this study, we aimed to gain more insight into the FPR signaling in regulating neutrophil functions, using pepducins as unique research tools. We found that a pepducin derived from the third intracellular loop of FPR1 inhibited neutrophils but the target was not FPR1. The FPR1 pepducin instead hijacked the closely related FPR2.

Abbreviations: CL, chemiluminescence; FPRs, formyl peptide receptors; PBP10, phosphoinositide binding peptide 10; GPCR, G-protein coupled receptor; KRG, Krebs–Ringer phosphate buffer; HRP, horseradish peroxidase; CysH, cyclosporin H; CR3, complement receptor 3

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2. Material and methods

2.1. Chemicals

Horseradish peroxidase (HRP), isoluminol, human recombinant TNFα, formylated tripeptide fMLF and C5a were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ficoll paque was obtained from GE Healthcare. The phenol-soluble modulin (PSMα2) in its N-formylated form was obtained from American Peptide Company (Sunnyvale, CA, USA) and the hexapeptides WKYMWM/m were purchased from AltaBioscience (University of Birmingham, Birmingham, U.K.). Compound 43 was a generous gift from Anygen. The receptor antagonist WRWWWW (WRW_4) was from Genscript Corporation (Scotch Plains, NJ, USA) and cyclosporin H (CysH) was kindly provided by Novartis Pharma (Basel, Switzerland). Phosphoinositide binding peptide 10 (PBP10) peptide (RhB-QRLFQVKGRR), MMK-1, fMIFL, the pepducins and the non-palmitoylated control peptide were obtained from Caslo Laboratory (Lyngby, Denmark). The pepducins were synthesized by Fmoc solid-phase peptide synthesis and the fatty acid were N-terminally linked on the resin as the last step before deprotection of side chains, followed by HPLC purification on a C18 column and further verification by MALTI-TOF Mass Spectrometry. The Cy5-WKYMWM peptide and FITC-fNLFNYK peptides were from Phoenix Pharmaceutical (Burlingame, CA). All peptides were dissolved in DMSO to a stock concentration of 10 mM and stored at −80 °C. Further dilutions were made in Krebs–Ringer phosphate buffer (KRG) supplemented with glucose (10 mM), Ca^{2+} (1 mM), and Mg^{2+} (1.5 mM) (KRG; pH 7.3).

2.2. Isolation of human neutrophils

Human polymorphonuclear neutrophils were isolated from buffy coats obtained from healthy adults as described [18]. Each independent experiment was performed on neutrophils isolated from an individual blood donor ($n > 3$). After hypotonic lysis, the neutrophils were washed twice, resuspended (1×10^7/mL) in KRG and, kept on melting ice until use.

2.3. Over-expression of FPR1 and FPR2 in HL-60 cells

The procedures used to obtain stable expression of FPR1 and FPR2 in undifferentiated HL-60 cells have been previously described [19]. At each passage, an aliquot of the cell culture was centrifuged, the supernatant was discarded and the cell pellet was resuspended in fresh medium RPMI 1640 containing FCS (10%), PEST (1%), and G418 (1 mg/ml).

2.4. Neutrophil NADPH-oxidase activity

The release of superoxide anion upon NADPH-oxidase activation was determined by an isoluminol-enhanced chemiluminescence (CL) system using a 6-channel Biolumat LB 9505 (Berthold Co, Wildbad, Germany) as described [20,21]. The neutrophils in measuring vials were pre-warmed for 5 min at 37 °C in the presence or absence of receptor inhibitors, after which the stimulus was added and superoxide production was recorded continuously.

2.5. Changes in the cytosolic concentration of free Ca^{2+}

Neutrophils freshly isolated from buffy coats (5×10^7/mL) or HL-60 cells overexpressing FPRs (5×10^7/ml) were labeled with Fura 2-AM (Molecular Probes, Eugene, OR, USA), and the transient rise in cytosolic Ca^{2+} concentration was determined using a PerkinElmer fluorescence spectrophotometer (LC50). For studies with inhibitors, the cells were pre-incubated with the inhibitors during the pre-warming period for 10 min before addition of the stimulus. The relative intracellular Ca^{2+} concentration is presented as the ratio between fluorescence intensities of the emitted light at 340 nm and 380 nm when excited at 510 nm.

2.6. Surface CR3 expression

The level of surface expression of CD11b/CR3 was determined using a phycoerythrin (PE)-conjugated antibody against CR3 (BD Biosciences, MD, USA). Cells in KRG (10^6/mL) were incubated with or without inhibitors at 37 °C for 5 min followed by stimulation with ligands and the incubation was continued for another 10 min. Cells were stained with antibody against CR3 or isotype control for 30 min on ice and binding analyzed by flow cytometry using an Accuri C6 (Becton Dickinson Sparks, MD, USA). Neutrophils were gated based on forward and side scatter.

2.7. Effect of the $F1Pal_{16}$ pepducin on FITC-fNLFNYK (FPR1 specific) and Cy5-WKYMVM (FPR2 specific) binding to neutrophils

Neutrophils in ice cold KRG (10^6/mL) were pre-incubated with unlabeled WKYMVM, or $F1Pal_{16}$ for 5 min before addition of the fluorescently labeled FPR2-specific agonist (Cy5-WKYMWM, 1 nM) or the FPR1-specific agonist (FITC-fNLFNYK, 1 nM) and the incubation was continued for another 60 min. The amount of bound peptide was analyzed by flow cytometry.

2.8. Statistical analysis

Statistical analysis was performed in Graph Pad Prism 6.0 (Graphpad Software, San Diego, CA, USA).

3. Results

3.1. No FPR1 related inhibition or activation is induced in neutrophils by the $F1Pal_{16}$ pepducin

Pepducins have been identified and characterized as either activating or inhibiting modulators in relation to their respective GPCR [7]. We have previously identified neutrophil-activating pepducins derived from FPR2, and the most potent pepducin was $F2Pal_{10}$, spanning the amino acids K_{227} to S_{236} (KIHKKGMIKS) in the third intracellular loop of FPR2 [13]. The third intracellular loop of FPR1 is very similar, differing in only two amino acids. We now confirm that the $F1Pal_{16}$ pepducin ($F1Pal_{16}$, Pal-KIHKQGLIKSSRPLRV; spanning the entire third intracellular loop of the receptor), in contrast to the corresponding FPR2 pepducin, lacked direct neutrophil activating effect, revealed by the inability of the pepducin to trigger superoxide anion production at concentrations up to 5 μM ([13], Fig. 1A inset). It is known that the level of superoxide production from neutrophils is largely dependent on the cellular state, with naïve neutrophils producing low levels of ROS and primed cells producing high levels of ROS [22], but not even neutrophils primed with TNFα produced superoxide upon stimulation with $F1Pal_{16}$ (data not shown).

We next took the inhibitor approach and investigated whether the $F1Pal_{16}$ pepducin could negatively modulate the FPR1-mediated response. The inhibitory effect of the established FPR1-antagonist CysH was confirmed (included as control with an FPR1 agonist in Fig. 1A). Conversely, $F1Pal_{16}$ did not affect the fMLF response (Fig. 1A). In summary, we show that $F1Pal_{16}$ has no modulating (neither activating nor inhibitory) effects related to FPR1 signaling.