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Similarities and differences between the responses induced in human phagocytes through activation of the medium chain fatty acid receptor GPR84 and the short chain fatty acid receptor FFA2R

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ABSTRACT

GPR84 is a recently de-orphanized member of the G-protein coupled receptor (GPCR) family recognizing medium chain fatty acids, and has been suggested to play important roles in inflammation. Due to the lack of potent and selective GPR84 ligands, the basic knowledge related to GPR84 functions is very limited. In this study, we have characterized the GPR84 activation profile and regulation mechanism in human phagocytes, using two recently developed small molecules that specifically target GPR84 agonistically (ZQ16) and antagonistically (GLPG1205), respectively. Compared to our earlier characterization of the short chain fatty acid receptor FFA2R which is functionally expressed in neutrophils but not in monocytes, GPR84 is expressed in both cell types and in monocyte-derived macrophages. In neutrophils, the GPR84 agonist had an activation profile very similar to that of FFA2R. The GPR84-mediated superoxide release was low in naïve cells, but the response could be significantly primed by TNFα and by the actin cytoskeleton disrupting agent Latrunculin A. Similar to that of FFA2R, a desensitization mechanism bypassing the actin cytoskeleton was utilized by GPR84. All ZQ16-mediated cellular responses were sensitive to GLPG1205, confirming the GPR84-dependency. Finally, our data of *in vivo* transmigrated tissue neutrophils indicate that both GPR84 and FFA2R are involved in neutrophil recruitment processes *in vivo*.

In summary, we show functional similarities but also some important differences between GPR84 and FFA2R in human phagocytes, thus providing some mechanistic insights into GPR84 regulation in blood neutrophils and cells recruited to an aseptic inflammatory site *in vivo*.

1. Introduction

Professional phagocytic neutrophils are key effector cells in inflammation, forming the first line of host defense against invading microbes [1,2]. Many basic functions of neutrophils including granule mobilization/secretion, migration and the release of reactive oxygen species (ROS) through the NADPH-oxidase, are regulated by surface expressed chemoattractant receptors belonging to the family of G-protein coupled receptors (GPCRs) [3]. Among the large number of receptors belonging to the GPCR family, neutrophils express e.g., two formyl peptide receptors (FPR1 and FPR2), the receptor for platelet activating factor (PAFR), those that recognize interleukin 8 (IL-8; CXCR1/2), complement fragment 5a (C5aR) and the danger signal ATP ($P2Y_2R$) as well as some members of the free fatty acid receptor group (FFARs) that has in common that they recognize free fatty acids (FFAs) of different carbon chain lengths [4–6].

During the past decade, an increasing number of orphan GPCRs have been de-orphanized, and these include the recently described FFARs sensing metabolic intermediates in the form of free fatty acids with different carbon chain lengths [7,8]. FFAs were traditionally

Abbreviations: APC, allophycocyaninin; AU, arbitrary units; BSA, bovine serum albumin; C5aR, complement fragment 5a receptor; CI, confidence interval; Cmp1, Compound 1; CTAB, Hexadecyltrimethylammonium bromide; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; FFAs, free fatty acids; FFARs, free fatty acid receptors; FITC, fluorescein; FPR, formyl peptide receptors; GPCR, G-protein coupled receptor; HRP, horseradish peroxidase; IL-8, interleukin 8; KRG, Krebs-Ringer phosphate buffer; M-CSF, macrophage colony-stimulating factor; MCFAs, medium chain fatty acids; MDMs, monocyte-derived macrophages; Mcpm, Mega counts per minute; PAFR, platelet activating factor receptor; PFA, paraformaldehyde; PE, phycoerythrin; PBMCs, peripheral blood mononuclear cells; PEST, Penicillin-Streptomycin; LA, Latrunculin A; PTX, pertussis toxin; RLU, relative light units; ROS, reactive oxygen species; SCFAs, short-chain fatty acids; TNFα, tumour necrosis factor α

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believed to exert their metabolic responses only through interactions with intracellular targets such as peroxisome proliferator-activated receptors [9], but it is now evident that FFAs facilitate their effects mainly through the group of GPCRs that together are termed FFARs. This receptor group includes FFA1R (earlier known as GPR40), FFA2R (earlier known as GPR43) and FFA3R (earlier known as GPR41) that have in common that they all bind short-chain fatty acids (SCFAs). Also, FFA4R (earlier known as GPR120) which binds both medium and long-chain fatty and *n*-3 polyunsaturated fatty acids (PUFA), and GPR84 (also known as EX33) sensing medium chain fatty acid (MCFAs) [6,8,10] belong to the family of FFARs. The levels of circulating FFAs has been shown to be elevated in several conditions associated with the metabolic disease syndrome e.g., liver cirrhosis and steatohepatitis, a link that highlights their role in metabolism [10,11]. Very recently, a direct connection was identified between the metabolic status and the function of the immune system [8], and in accordance with this, FFARs have gained increased interest also for potential roles as modulators of immune cell functions and the outcome of inflammatory reactions. In line with this, by employing the recently identified FFA2R selective agonist Compound 1 (Cmp1) and antagonist CATPB, we and others have provided data on the functional expression and modulation of the short chain fatty acid binding FFA2R expressed in human neutrophils [4,12]. In this study, we show that the activation/deactivation pattern of FFA2R is in some aspects different from that of the formyl peptide receptors (FPRs), the most studied pattern recognition receptors expressed in phagocytes and the prototypical neutrophil GPCRs [4]. These findings have now been expanded to include GPR84, a FFAR that was de-orphanized in 2006 and that has been shown to recognize MCFAs with chain lengths of 9– 14 carbon atoms [13]. Compared to the extensively studied FPRs and the recent characterized FFA2R, very little is known about the activation pattern and regulation mechanisms of GPR84 in neutrophils except for the fact that the receptor is highly expressed by peripheral blood leukocytes [6]. Basic characterization of the activation pattern of GPR84, has been hindered by the lack of potent and selective molecular tools, a problem still common and shared with many other GPCRs. Molecular tools that selectively target GPR84 have, however, recently become available.

To characterize the basic functions of GPR84 in phagocytes, we have utilized the receptor selective antagonist GLPG1205 (a small selective antagonist of GPR84 developed by Galapagos NV [14]) together with ZQ16 (2-(hexylthio) pyrimidine-4,6-diol), a potent GPR84 selective agonist identified in a high throughput screening study [15]. We show that GPR84 is functionally expressed by human neutrophils, monocytes and monocyte-derived macrophages (MDMs). The GPR84 selective agonist triggered the G-protein coupled PLC-IP_3-Ca^{2+} signaling pathway, mobilization of intracellular granules, chemotactic migration, as well as an assembly of ROS generating NADPH-oxidase. The receptor selectivity was confirmed as the ZQ16 induced responses were inhibited by the GPR84 selective antagonist GLPG1205. The very low release of ROS induced by the GPR84 agonist in naïve neutrophils was significantly increased by pre-treatment of neutrophils with the pro-inflammatory priming agent tumour necrosis factor α (TNFα). Although the ZQ16 induced response was augmented in neutrophils with a disrupted actin cytoskeleton, GPR84 signaling was terminated by a cytoskeleton independent mechanism. In summary, our results demonstrate that the GPR84 antagonist GLPG1205 selectively inhibits the function of human professional phagocytes activated with the GPR84 agonist ZQ16. We also provide data revealing some mechanistic insights into GPR84-mediated immune modulation related to activation and termination of phagocyte functions, which shows similarities as well as differences to those of FFA2R.

2. Materials and methods

2.1. Ethics statement

This study, conducted at the Sahlgrenska Academy in Sweden, includes blood from buffy coats obtained from the blood bank at Sahlgrenska University Hospital, exudated tissue neutrophils, and peripheral blood from healthy donors. Regarding exudated tissue neutrophils and peripheral blood from healthy donors, the study was approved by the ethical committee in Gothenburg, Sweden (Sweden; No. 543-07). According to the Swedish legislation section code 4§ 3p SFS 2003:460 (Lag om etikprövning av forskning som avser människor), no ethical approval was needed for the buffy coats since the buffy coats were provided anonymously and could not be traced back to a specific donor.

2.2. Chemicals and reagents

Dextran and Ficoll-Paque were obtained from GE-Healthcare Bio-Science (Uppsala, Sweden). Horseradish peroxidase (HRP) and catalase was obtained from Boehringer Mannheim and fetal calf serum (FCS), Penicillin-Streptomycin (PEST), human recombinant IL-8 and macrophage colony-stimulating factor (M-CSF) were from R&D Systems. RPMI 1640 culture medium with and without phenol red were from PAA Laboratories GmbH (Pasching, Austria). Isoluminol, Latrunculin A (LA), pertussis toxin (PTX), tumour necrosis factor α (TNFα), Hexadecyltrimethylammonium bromide (CTAB), capric acid/decanoic acid ($C_{10}H_{20}O_2$), undecanoid acid ($C_{11}H_{22}O_2$), dimethyl sulfoxide (DMSO), Giemsa, and phorbol 12-myristate 13-acetate (PMA) were from Sigma. May-Gründwald was from Kebolab. WKYMVM was synthesized by CASLO Laboratory (Lyngby, Denmark). Fura-2-AM was from Life Technologies Europe (Stockholm, Sweden). Fluo-3-AM and FuraRed-AM were from Invitrogen Molecular Probes. The CD45 antibody conjugated with allophycocyaninin (APC) was from Abcam and the phycoerythrin (PE)-conjugated and APC-conjugated antibodies against CD62L, CD35 and CD11b, and FACS Lysing Solution were from Becton Dickinson Biosciences. The fluorescein (FITC)-conjugated antibody against CD66b was from AbD Serotec/Bio-Rad and paraformaldehyde (PFA) was from Fischer Scientific. YM-254890 was purchased from WAKO (Wako Chemicals GmbH, Neuss, Germany). The GPR84 agonist ZQ16 was obtained from Tocris Bioscience and GLPG1205, a potent and selective antagonist of GPR84 was kindly provided by Galapagos NV (more information about Galapagos NV at http://www.glpg.com) [14].

All peptides were dissolved in DMSO and stored at −80 °C until use. Subsequent dilutions of all reagents were made in Krebs-Ringer phosphate buffer (KRG, pH 7.3; 120 mM NaCl, 5 mM KCl, 1.7 mM KH_2PO_4, 8.3 mM NaH_2PO_4 and 10 mM glucose) supplemented with Ca^{2+} (1 mM) and Mg^{2+} (1.5 mM).

2.3. Isolation of peripheral blood neutrophils and monocyte-derived macrophages

Human peripheral blood neutrophils were isolated from buffy coats or from freshly drawn blood of healthy blood donors using dextran sedimentation and Ficoll-Paque gradient centrifugation as described [16]. The remaining erythrocytes were disrupted by hypotonic lysis and the neutrophils were washed, re-suspended in KRG, and stored on melting ice until use. This isolation procedure permits cells to be purified with minimal granule mobilization. Macrophages were differentiated from monocytes isolated from buffy coats as described [17]. Briefly, the peripheral blood mononuclear cells (PBMCs) were diluted in RPMI-1640 with 10% FCS and 1% PEST to a concentration of 4×10^6 cells/mL and cultured (2 mL/well) on 12-wells polystyrene plates (tissue cultured treated by vacuum gas plasma from Fischer Scientific) in the presence of M-CSF (15 ng/mL). The medium was