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5-Oxo-Eicosatetraenoic Acid-Induced Chemotaxis: Identification of a Responsible Receptor hGPCR48 and Negative Regulation by G Protein $G_{12/13}$

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While screening genes encoding G protein–coupled receptors (GPCRs) in the human genome, we and other groups have identified a GPCR named hGPCR48 as a high affinity receptor for 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), which is arachidonic acid metabolite and an endogenous chemoattractant for granulocytes. Using Chinese hamster ovary (CHO) cells stably expressing hGPCR48, we show here that activation of the receptor causes the chemotaxis of the cells toward 5-oxo-ETE. We also show that the chemotaxis of human granulocytes toward 5-oxo-ETE is inhibited by pretreatment with anti-hGPCR48 antibodies, indicating that hGPCR48 is an endogenous receptor responsible for chemotaxis of granulocytes toward 5-oxo-ETE. In addition, we show that the chemotaxis of CHO cells expressing hGPCR48 is suppressed by pretreatment with pertussis toxin, and enhanced by overexpression of the carboxy terminal peptides of $G\alpha_{12/13}$ subunits or a regulator of the G protein signaling domain of p115RhoGEF, both of which are known to suppress $G_{12/13}$-dependent signaling pathways. These results indicate that hGPCR48 couples with $G_{i/o}$ and $G_{12/13}$ proteins, which then initiate or attenuate the chemotaxis of the cells toward 5-oxo-ETE, respectively.

Key words: chemotaxis, G protein-coupled receptor, C12/13, 5-oxo-ETE.

Abbreviations: IBMX, 3-isobutyl-1-methylxanthine, 5-HETE, 5(*S*)-hydoxy-eicosatetraenoic acid, 5-HPETE, 5(*S*)-hydroperoxyeicosatetraenoic acid, 5-oxo-ETE, 5-oxo-6,8,11,14-eicosatetraenoic acid, BSA, bovine serum albumin, G12-ct/G13-ct, carboxyl terminal regions of $G_{12/13}$ α subunit, CHO, Chinese hamster ovary, GPCR, G-protein–coupled receptor, HTB, Hepes Tyrode's bovine albumin buffer, PTX, pertussis toxin, PBS, phosphate buffered saline; PCR, polymerase chain reaction, RGS, regulator of G protein signaling, S1P, sphingosine-1-phosphate.

Granulocytes play an important role in many allergic reactions (*1*). The migration of granulocytes into inflammatory sites is mediated by several bioactive peptides and lipid mediators that are known as chemoattractants. 5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), a metabolite of arachidonic acid, has been reported to show a potent activity for chemotaxis of granulocytes such as neutrophils (*2*–*5*), eosinophils (*4*, *6*–*8*), and monocytes (*9*), and to play a role in the inflammatory response. These responses to 5-oxo-ETE of human granulocytes are sensitive to pertussis toxin (PTX), which is known to uncouple the interaction between G protein–coupled receptors (GPCRs) and G proteins $G_{i/o}$ (*10*, *11*). These findings suggest that the stimulation by 5-oxo-ETE is mediated *via* a $G_{i/o}$-coupled GPCR. Recently, three independent groups, including ours, identified a GPCR with a high affinity for 5-oxo-ETE that was termed as hGPCR48 (*12*), TG1019 (*13*), or R527 (*14*) by each group; hereafter this receptor is referred to as hGPCR48. The character of hGPCR48 is summarized as follows: (i) A fusion protein of hGPCR48 with G protein α_{i1} subunit is activated by 5-oxo-ETE with an EC_{50} value of 5.5–5.7 nM, as assessed by agonist-stimulated [^{35}S]GTPγS binding activity; the EC_{50} value is similar to the value reported for its binding with membrane preparations of neutrophils (*12*, *13*). (ii) Treatment with PTX inhibits the 5-oxo-ETE–induced decrease in the cAMP content in forskolin-stimulated cultured cells expressing hGPCR48 (*13*). (iii) hGPCR48 is highly expressed in granulocytes as revealed by RT-PCR analysis (*14*). All these findings are consistent with the idea that hGPCR48 is a receptor responsible for 5-oxo-ETE–induced chemotaxis of granulocytes. However, no direct evidence has yet been presented.

G proteins are divided into four families based on the homology of their amino acid sequences: G_s, G_i, G_q, and G_{12} (*15*, *16*). The G_{12} family, which composes two members, G_{12} and G_{13}, is the most recently identified and is ubiquitously expressed. It is not well understood, however, which kinds of GPCRs are coupled with the $G_{12/13}$ family. Recently, $S1P_3$, a subtype of a sphingosine-1-phosphate (S1P) receptor and another receptor for cell chemotaxis, was found to be coupled with both G_i and $G_{12/13}$. A $S1P_3$ receptor has

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been reported to mediate the G_i-dependent activation of Rac and cell migration in response to S1P. On the other hand, these responses are inhibited by $G_{12/13}$-pathways (*17*, *18*). It is of interest to know whether the involvement of $G_{12/13}$ as well as G_i is generalized for other type of chemotaxis. Therefore, we have examined whether $G_{12/13}$ is involved in the chemotaxis in response to 5-oxo-ETE.

In this paper, we provide evidence that hGPCR48 is involved in 5-oxo-ETE–induced chemotaxis; hGPCR48, heterologously expressed in CHO cells, mediates chemotaxis in response to 5-oxo-ETE, and an anti-hGPCR48 antibody inhibited the migration of granulocytes to 5-oxo-ETE. Furthermore, we provide evidence that $G_{12/13}$ is negatively involved in the 5-oxo-ETE–induced chemotaxis by using a carboxyl terminal portion of $G_{12/13}$ (*19*) and a regulator of the G protein signaling (RGS) domain of p115RhoGEF (*20*), which selectively interact with $G_{12/13}$ thus interfering with its interaction with the receptor.

MATERIALS AND METHOD

Materials—5-Oxo-ETE, 5(*S*)-hydoxyeicosatetraenoic acid (5-HETE) and 5(*S*)-hydroperoxyeicosatetraenoic acid (5-HPETE) were purchased from BIOMOL. The protease inhibitor mixture and PTX were purchased from Sigma. Dulbecco's modified Eagle's medium and Hanks' balanced salt solution were purchased from Invitrogen. The stock solution of Hepes Tyrode's bovine albumin buffer (HTB) contains 125 mM Hepes/NaOH (pH 7.4), 700 mM NaCl, 13.5 mM KCl, 60 mM $NaHCO_3$, 2 mM NaH_2PO_4, 2.5 mM $MgCl_2$, 5 mM $CaCl_2$, 28 mM D-glucose, 0.05% bovine serum albumin (BSA). This stock solution was diluted 5 times just before use.

Construction of Expression Vectors—The coding sequence of hGPCR48 (*12*) was amplified by PCR from human genomic DNA (TOYOBO) with 5′-AAAGCGGCCG-CATGTTGTGTCACCGTGGTGG-3′ (forward primer) and 5′-AAATCTAGATCAGCCCTGGGAGGAGCCTT-3′ (reverse primer). The PCR reaction was performed using 1 unit of KOD plus polymerase (TOYOBO), 5 μM each of forward and reverse primer, 1 mM $MgCl_2$, 1 mM dNTPs, 0.1 μg genomic DNA, 1× buffer supplied by the manufacturer, and a temperature program of 98°C, 15 s, 60°C, 15s, 68°C, 2 min, for 30 cycles. The PCR products were purified by 1% agarose gel electrophoresis and cloned into the *Not*I–*Xba*I sites of pcDNA3.1 (Invitrogen). The carboxyl terminal regions of G_{12} or G_{13} (G12-ct/G13-ct) were cloned from the mouse genomic DNA (TOYOBO) by using PCR with KOD plus polymerase (TOYOBO) and two pairs of primers: 5′-TTTGGATCCATGGTCCAGCGCTACCTGGTGC-3′ (forward primer) and 5′-TTTCTCGAGTCACTGCAGCATGAT-GTCTTTCA-3′ (reverse primer) for G_{12} (G12-ct, amino acids 335–379); 5′-TTTGGATCCATGGTCCAAAAGTTT-CTGGTGGA-3′ (forward primer) and 5′-TTTCTCGAGT-CACTGCAGCATGAGCTGCT-3′ (reverse primer) for G_{13} (G13-ct, amino acids 322–377) (*19*). The amino terminal region containing the RGS domain of p115RhoGEF (p115-RGS, amino acids 1–252) was amplified with a template plasmid of a p115-RGS construct that was a kind gift from Professor Tohru Kozasa (University of Texas) and a pair of primers; 5′-TTTGGATCCATGGAAGACTTCGCCC-GAGG-3′ (forward primer) and 5′-TTTCTCGAGGTTCCC-CATCACCTTTTTCCG-3′ (reverse primer) (*20*). The temperature program was 30 cycles of 30 sec at 94°C, 15 s at 60°C and 2 min at 68°C. The PCR products were purified by 1.5% agarose gel electrophoresis and inserted into pcDNA-4/HisMaxB (Invitrogen) between the *Bam*HI and *Xho*I sites. The DNA sequences of all constructs were confirmed by a DNA sequence ABI310 analysis system (Applied Biosystems).

Cell Culture, Transfection, and Cell Cloning—CHO cells were grown in Ham's F-12 supplemented with 10% fetal bovine serum under 5% CO_2 at 37°C. Transient transfection with expression vectors was carried out by using LipofectAMINE (Invitrogen) for 48 hour according to the manufacturer's protocol. To establish CHO cells that stably expressing hGPCR48, cells were selected in the presence of 0.4 mg/ml of G418 (Nacalai), and cloned by the standard limiting dilution method. CHO cells stably expressing hGPCR48 were maintained in the presence of 0.1 mg/ml G418. PTX treatment was carried out by incubating cells in serum-free F-12 medium containing 100 ng/ml PTX for 24 h before experiments.

cAMP Assay—For measurements of adenylate cyclase activity, cells were seeded in 96-well plates (1.5×10^4 cells/well) and incubated at 37°C in 5% CO_2. After 24 h, the cells were washed twice with HTB and incubated in HTB containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 10 min. After these treatments, the cells were stimulated with a drop of ligand solution and incubated in the presence of 5 μM forskolin at 37°C under 5% CO_2 for 30 min. Intracellular cAMP concentrations were measured using a cAMP-Screen System (Applied Biosystems) and a Wallac 1420 luminometer (Perkin Elmer).

Calcium Mobilization Assays—Intracellular calcium ion levels were determined by measuring the fluorescence of Fura-2. Cells expressing hGPCR48 were suspended at a final concentration of $1–5 \times 10^6$ cells/ml in loading buffer containing 2.5 μM Fura-2/AM (Dojin), 1.25 mM probenecid (Wako) to inhibit anion transporters, and 0.02% F-127 (Molecular Probes) to avoid aggregation of the Fura-2/AM, and incubated at 37°C under 5% CO_2 for 1 h to load the Fura-2. After washing the cells with HTB, intracellular calcium ion levels were measured following the addition of 6 μl ligand solution into 600 μl of cell suspension using an RF-5300PC spectrofluorometer system (Shimadzu).

Chemotaxis Assay—A polycarbonate filter with 5 μm pores (Neuroprobe) was coated with 10 μg/ml fibronectin (Wako) in phosphate buffered saline (PBS) for 30 min, and placed in a 96-well Boyden chamber (Neuroprobe). We added ligand droplets (34 μl/well) to the bottom wells and CHO cells (200 μl, 8×10^4 cells/well) to the top wells of the chamber. The ligand solution and cell suspension were prepared in the serum-free Ham's F-12 medium supplemented with 0.1% fatty acid–free BSA (Serologicals). After incubation at 37°C under 5% CO_2 for 4 h, the Boyden chamber was disassembled. The cells on the filter were fixed with methanol and stained with a Diff-Quick staining kit (International Reagent Corporation). The upper side of the filter was scraped to remove the cells. The number of the cells that migrated to the lower side was determined by measuring the optical density at 595 nm using a 96-well microplate reader (Bio-Rad Model 680).

Antibody Preparation and Purification—The putative outer membrane region of hGPCR48 (amino acids 22–92

for the first outer membrane loop) was amplified with a template plasmid carrying the hGPCR48 gene and two primers, 5′-TTTGGATCCTGCAGGGGTAGAAGACTCCA-GAAC-3′ (forward primer) and 5′-AAACTCGAGAGACAC-CAGCGAGGAAGAGGT-3′ (reverse primer) using KOD plus polymerase (TOYOBO). The temperature program was 30 cycles of 30 s at 94°C, 15 s at 60°C and 120 s at 68°C. The amplified DNA was purified by 1.5% agarose gel electrophoresis and inserted into pGEX-4T-3 (Amersham) between the *Bam*HI–*Xho*I sites. *E. coli* BL21(DE3) cells were transformed with this expression vector, and grown at 28°C in Luria broth with 100 μg/ml ampicillin. Isopropyl-1-thio-β-D-galactosidase was added at a concentration of 1 mM when the optical density at 600 nm reached 0.45. The cells were continuously cultivated for 3 h afterwards, and then harvested by centrifugation at 6,000 rpm for 10 min. The resulting cell pellet was re-suspended in PBS containing 100 μg/ml lysozyme and sonicated on ice. The extract was centrifuged at 6,000 rpm for 10 min to remove cell debris. The supernatant of the lysed cells was loaded onto a glutathione Sepharose 4B (Amersham) column equilibrated with PBS, and proteins were eluted in the presence of 10 mM glutathione. Fractions for the fusion protein of glutathione S-transferase and the first outer membrane loop of hGPCR48 were concentrated using Amicon ultrafiltration cells with YM-10 filters (Millipore) and dialyzed twice against PBS. All protein fractions were monitored for absorption at 280 nm, and examined by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Purified antigen was injected into a rabbit in Freund's complete adjuvant. The obtained anti-serum was applied onto antigen-conjugated Sepharose equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The antibody was eluted by 1M HCl and dialyzed againsr PBS. The specificity of the antibody was confirmed by western blotting (data not shown) using Sf9 cell membranes that expressing the hGPCR48-G_i fusion protein (*12*).

Preparation of Human Granulocytes and Transwell Assay—Human granulocytes were isolated from human peripheral blood using Polymorphprep (Daiichi Pure Chemicals) according to the manufacturer's protocol. Briefly, 20 ml of freshly isolated venous blood from healthy volunteers was treated with heparin, applied to 20 ml of Polymorphprep and centrifuged for 30 min at 500 × *g* at 20°C. The cells were collected from each interface and contaminating erythrocytes were lysed by hypotonic shock. Thereafter, the supernatant containing granulocytes was diluted with PBS and centrifuged for 10 min at 500 × *g* at 20°C. The sedimented cells were washed three times and suspended in HTB. The prepared granulocytes (2×10^6 cells/ml) were pre-incubated in the presence or absence of 15 μg/ml anti-hGPCR48 antibody at 37°C, 10 min. Chemotaxis assays for human granulocytes were performed in 24-well Transwell plates with an 5 μm pore-size polycarbonate filters (Costar). HTB containing 5-oxo-ETE was placed in the lower compartment, and the granulocytes (2×10^5 cells) were placed in the upper compartment. The cells were then incubated for 2 h at 37°C under 5% CO_2. Cell migration was determined by counting cells that had migrated through the filter using a Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) and a Wallac 1420 luminometer (Perkin Elmer).

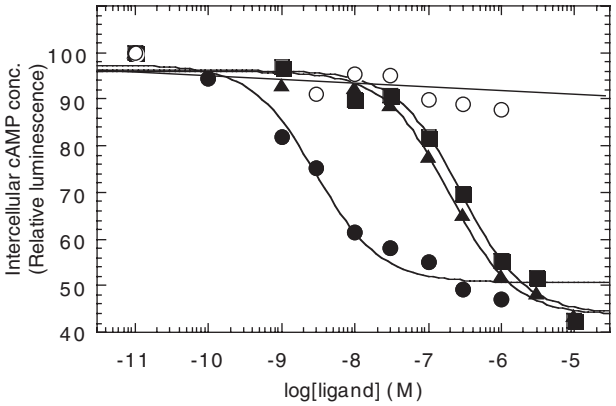

Fig. 1. **For cAMP assay, cells were seeded in 96-well plates (1.5×10^4 cells/well) and incubated under 5% CO_2 at 37°C for 24 h.** The cells were pretreated with 1 mM IBMX for 10 min and then stimulated with 5-oxo-ETE (filled circles), 5-HPETE (squares), or 5-HETE (triangles) for 30 min in the presence of 5 μM forskolin. Intracellular cAMP concentrations were measured using a cAMP-Screen System (Applied Biosystems) and a Wallac 1420 luminometer (Perkin Elmer). The EC_{50} values for 5-oxo-ETE, 5-HPETE, and 5-HETE were 2.9 ± 0.76 nM, 280 ± 66 nM, 190 ± 33 nM, respectively. The activity induced by 5-oxo-ETE was suppressed by PTX pretreatment (open circles).

RESULTS

Stable Expression of hGPCR48 in CHO Cells and the Receptor-Mediated Changes in Second Messengers—A stable cell line expressing hGPCR48 was established to study the involvement of the hGPCR48 in chemotaxis. An expression construct containing the coding region of hGPCR48 gene was used for transfection, and cells were cloned by G418 selection and limiting dilution. Expression of hGPCR48 mRNA was confirmed by RT-PCR (data not shown). The function of the expressed hGPCR48 was confirmed by measuring 5-oxo-ETE–induced changes in second messengers.

5-oxo-ETE and two related lipids inhibited cAMP formation in forskolin-stimulated cells in a dose-dependent manner (Fig. 1). The IC_{50} for 5-oxo-ETE, 5-HPETE, and 5-HETE were estimated to be 2.9 ± 0.76 nM, 280 ± 66 nM, 190 ± 33 nM, respectively. These three compounds functioned as full agonists. Increases in intracellular calcium concentrations were also observed following stimulation with 5-oxo-ETE and 5-HPETE, and the EC_{50} for 5-oxo-ETE and 5-HPETE were estimated to be 1.2 ± 0.5 nM and 16 ± 5.8 nM, respectively (Fig. 2). In contrast to the effect on cAMP content, 5-HPETE functioned as a partial agonist and increased the intracellular calcium concentration up to 70% of the value attained with 5-oxo-ETE. The efficacy of 5-HETE was much lower and only a very weak effect was observed even at a concentration of 1 μM. The agonist-induced inhibition of adenylate cyclases (Fig. 1) and increase in intracellular calcium ion levels (Fig. 2) were suppressed by preincubating the cells with PTX, confirming that the hGPCR48 is coupled with G protein $G_{i/o}$ (data not shown).

5-Oxo-ETE–Induced Chemotaxis of CHO Cells Expressing hGPCR48—The chemotaxis of CHO cells expressing hGPCR48 was examined using a Boyden chamber. Cell accumulation was induced by 5-oxo-ETE and

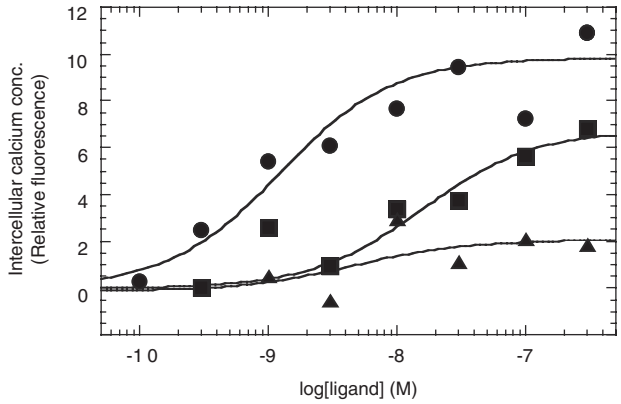

Fig. 2. **Intracellular calcium ion levels were measured using a calcium ion sensitive fluorescence dye, Fura-2.** Cells expressing hGPCR48 were suspended in 1.25 mM probenecid, 0.02% F-127, and 2.5 μM Fura-2/AM at a final concentration of 1–5 × 10^6 cells/ml, and incubated at 37°C under 5% CO_2 for 1 h. The cell suspension (600 μl) was stimulated by adding 6 μl of 5-oxo-ETE (circles), 5-HPETE (squares), and 5-HETE (triangles) and then the fluorescence was measured by a RF-5300PC spectrofluorometer (Shimadzu). The EC_{50} values for 5-oxo-ETE and 5-HPETE were estimated to be 8.7 × 6.7 nM and 960 × 780 nM, respectively.

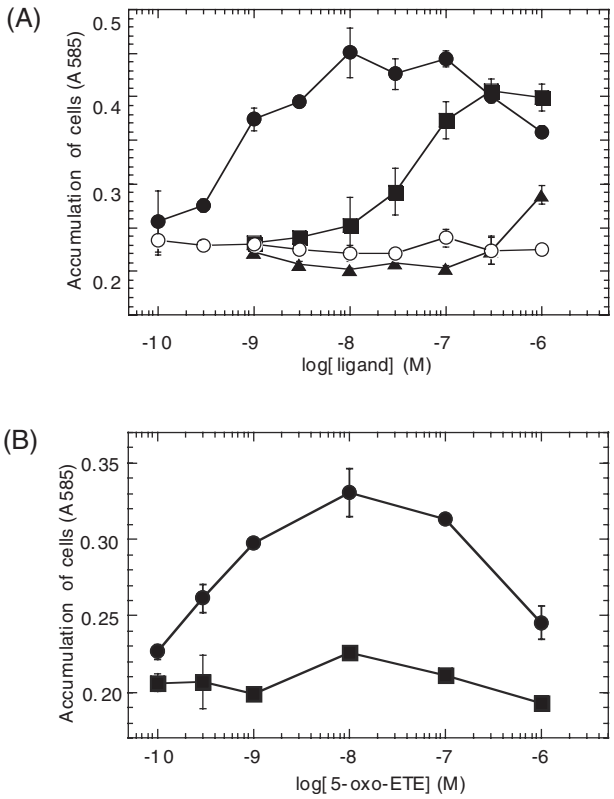

Fig. 3. **(A) The cell migration was measured using a Boyden chamber and a polycarbonate filter with 5 μm pores coated with fibronectin in the presence of 5-oxo-ETE (filled circles), 5-HPETE (squares), and 5-HETE (triangles) in the bottom chamber.** The EC_{50} values were estimated to be 0.6 ± 0.17 nM and 42 ± 12 nM for 5-oxo-ETE and 5-HPETE, respectively. The chemotaxis toward 5-oxo-ETE was suppressed by PTX pretreatment (open circles). (B) Cell migration was measured as in (A) for chemotaxis (circles). The same procedure was applied except that 5-oxo-ETE was present in both the top and bottom chambers for chemokinesis (squares). The cells were incubated at 37°C under 5% CO_2 for 4 h for migration. Then, the cells on the filter were stained, and their densities were estimated by measuring the optical densities at 595 nm with a 96-well microplate reader (Bio-Rad Model 680).

5-HPETE in a dose-dependent manner, although the chemoattracting activity of 5-HETE was not clear. The EC_{50} values were estimated to be 0.6 ± 0.17 nM for 5-oxo-ETE and 42 ± 12 nM for 5-HPETE (Fig. 3A). The cells did not migrate to the lower side of the filter when attractants were present on both sides of the filter (Fig. 3B). This confirms that the accumulation of cells on the filter represents chemotaxis toward attractants, and not chemokinesis, that is, the stimulation of cell movement by ligands. No chemotaxis toward 5-oxo-ETE was observed in control cells transfected with an empty vector (data not shown) or in hGPCR48-expressing cells pretreated with PTX (Fig. 3A). We, therefore, conclude that the activation of hGPCR48 by 5-oxo-ETE and the following activation of G protein $G_{i/o}$ induced the chemotaxis toward 5-oxo-ETE.

Inhibition of 5-Oxo-ETE–Induced Chemotaxisis of Human Granulocytes by Anti-hGPCR48—Human granulocytes are known to migrate toward 5-oxo-ETE in a PTX-sensitive manner (*10*) and to express the mRNA (*14*) for hGPCR48. These facts and the results of hGPCR48-expressing CHO cells presented in Fig. 3 strongly suggest that hGPCR48 mediates the chemotaxis of human granulocytes toward 5-oxo-ETE, but no direct evidence has yet been presented. Therefore, we examined the effects of anti-hGPCR48 antibodies on the chemotaxis of human granulocytes toward 5-oxo-ETE. Although the extent of chemotaxis and the EC_{50} values for 5-oxo-ETE tend to differ from one guanulocyte preparation to another, inhibitory effect of anti-hGPCR48 antibodies on chemotaxis was consistently observed. Figure 4 shows the effects of the antibodies on chemotaxis of granulocytes in the presence of different concentrations of 5-oxo-ETE: the EC_{50} was estimated to be 180 nM in this experiment. The addition of antibodies reduced the number of granulocytes accumulating in response to 100 nM 5-oxo-ETE to approximately 25% of the amount in the absence of the antibodies (Fig. 4). In contrast, the same treatment with anti-hGPCR48 antibodies hardly affected the chemotxis of granulocytes toward LTB_4, which is the other potent chemoattractant derived from arachidonic acid (*21*) (data not shown). These results indicate that the chemotaxis of human granulocytes toward 5-oxo-ETE is mediated through hGPCR48.

Involvement of G Proteins $G_{12/13}$ in the Chemotaxis of hGPCR48-Expressing CHO Cells—We have examined whether G proteins $G_{12/13}$ are involved in the chemotaxis of the hGPCR48-expressing CHO cells by using carboxyl terminal peptides of $G_{12/13}$ (G12-ct or G13-ct), which are expected to selectively inhibit the receptor-$G_{12/13}$ interaction (*19*). CHO cells stably expressing hGPCR48 cells were transfected with cDNA encoding G12-ct or G13-ct, and then subjected to chemotaxis experiments. The chemotaxis activities in response to 5-oxo-ETE were increased in the cells transfected with G12-ct (Fig. 5A) or G13-ct (Fig. 5B) expression vector compared to cells transfected with empty vector. It should be noticed that higher concentrations of

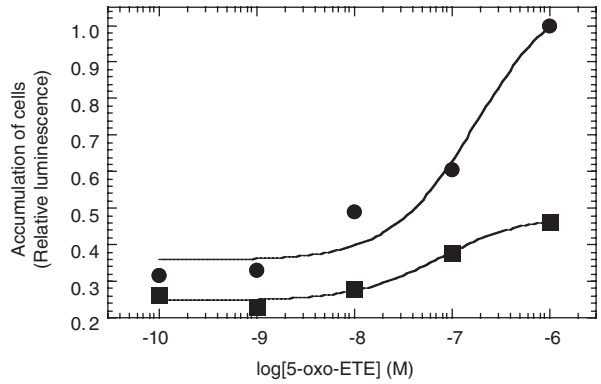

Fig. 4. **Inhibition of granulocyte migration toward 5-oxo-ETE by anti-hGPCR48 antibody.** Human granulocytes were isolated by using Polymorphprep (Daiichi Pure Chemicals). The granulocytes were pre-incubated in the presence (squares) or absence (circles) of 15 μg/ml anti-hGPCR48 antibody at 37°C for 10 min. The granulocytes (2×10^5 cells) were placed in the upper compartments with or without anti-hGPCR48 antibody, and 1 μM 5-oxo-ETE was placed in the lower compartments of 24-well Transwell plates with a 5 μm pore-size polycarbonate filter. The cells were then incubated for 2 h at 37°C in 5% CO_2. Cell migration was determined by counting cells that had migrated through the filters using a Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) and a Wallac 1420 luminometer (Perkin Elmer).

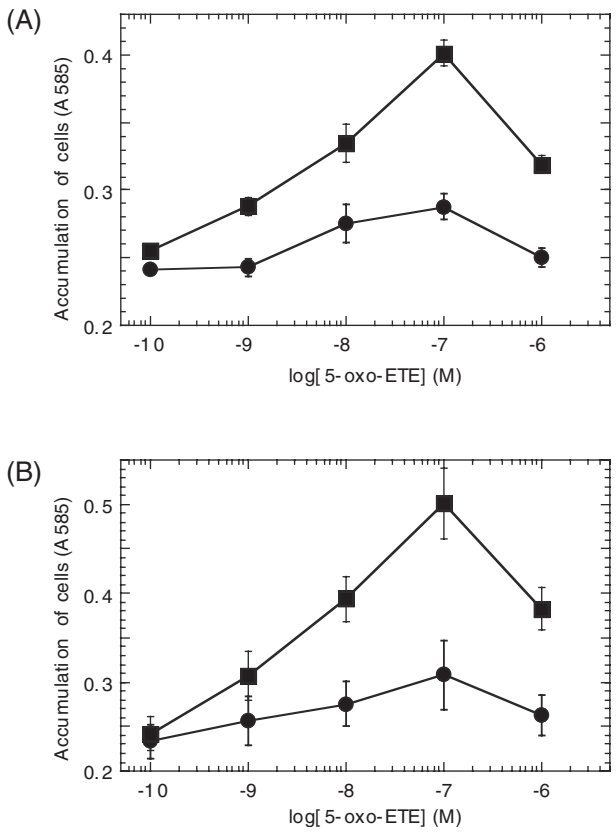

Fig. 5. **Effects of the overexpression of G12-ct (A) and G13-ct (B) on chemotaxis of CHO cells expressing hGPCR48.** CHO cells stably expressing hGPCR48 were transiently transfected with an expression vector for G12-ct (A) or G13-ct (B) (squares) or an empty vector (circles), then used for chemotaxis experiments after 48 h. The cell migration assay was performed as described in the legend to Fig. 3.

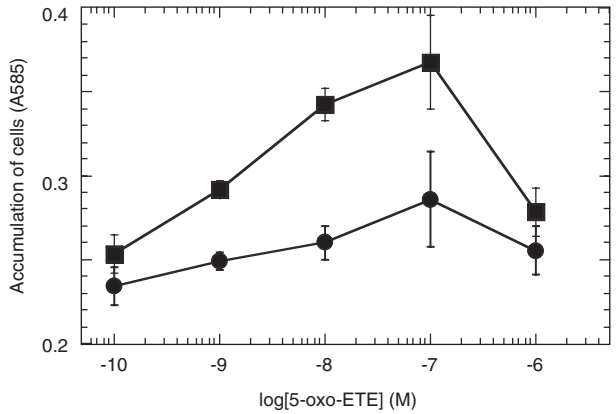

Fig. 6. **Effects of the overexpression of p115-RGS on the chemotaxis of CHO cells expressing hGPCR48.** Transient transfections with an expression vector for p115-RGS (squares) or empty vector (circles) and the cell migration assay were carried out as described in the legend to Fig. 3.

5-oxo-ETE were required for chemotaxis of transiently transfected cells. The EC_{50} was approximately 10 nM for the transfected cells in contrast to 0.6 nM for non-transfected cells (Fig. 3). The number of migrating CHO cells was also reduced after transfection. These effects were observed even with transfection of an empty vector, alone and might be due to side effects of LipofectAMINE treatment. Despite of these unexpected effects, the expression of G12-ct or G13-ct clearly increased the extent of cell migration in response to 5-oxo-ETE. To further confirm the involvement of $G_{12/13}$ in chemotaxis in response to 5-oxo-ETE, we expressed the $G_{12/13}$-selective RGS domain of p115 RhoGEF (p115-RGS), which is also expected to suppress $G_{12/13}$ signaling (*20*). As shown in Fig. 6, the transient expression of p115-RGS significantly enhanced 5-oxo-ETE–induced chemotaxis. These results indicate that hGPCR48 interacts not only with $G_{i/o}$ but also with $G_{12/13}$, and that the interaction of hGPCR48 with $G_{12/13}$ results in the suppression of cell migration, whereas its interaction with $G_{i/o}$ leads to an initiation of chemotaxis.

DISCUSSION

A GPCR termed as hGPCR48, TG1019, or R527 has been shown to have high affinity for 5-oxo-ETE (5.5–5.7 nM of EC_{50} as assessed by the 5-oxo-ETE–induced increase in [^{35}S]GTPγS binding of hGPCR48-$G_{i1}\alpha$ fusion protein) (*12*, *13*). Granulocytes including eosinophils and neutrophils have been shown to migrate toward 5-oxo-ETE through the activity of a putative GPCR linked to G proteins $G_{i/o}$ (*10*). The present paper provides evidence that hGPCR48 is involved in chemotaxis toward 5-oxo-ETE. We stably transfected the hGPCR48 gene into CHO cells and confirmed that the receptor can mediate ligand-induced signals in a PTX-sensitive manner, as assessed by ligand-induced changes in second messengers. 5-Oxo-ETE was a most potent ligand, followed by 5-HPETE and 5-HETE. The EC_{50} value for 5-oxo-ETE was estimated to be 1.2 nM for intracellular calcium ion increase, which is comparable to the value of 2 nM reported for the intracellular calcium ion increase in human neutrophils

stimulated by 5-oxo-ETE (*2*). This result supports the idea that the response of neutrophils is mediated by hGPCR48. During the preparation of this paper, Hosoi *et al.* (*22*) also reported the expression of hGPCR48 (TG1019 by their designation) in CHO cells, and gave a similar EC_{50} value of 5.1 nM for the intracellular calcium ion response. Jones *et al*. (*14*), however, reported a higher EC_{50} value (115 nM) for the 5-oxo-ETE-induced increase in intracellular calcium ion using HEK293 cells expressing R527, an N-terminus–truncated form of hGPCR48. Although the reason for this discrepancy remains unknown, it may be due to the difference in the cells used, the presence of coexpressed G_{16}, or the N-terminus–truncated form of the receptor used in their experiments.

We show here that hGPCR48-expressing CHO cells, but not control CHO cells, migrate toward 5-oxo-ETE, 5-HPETE, or 5-HETE, with a potency and efficacy in the order of 5-oxo-ETE > 5-HPETE > 5-HETE. The chemotaxis toward 5-oxo-ETE was completely inhibited by pretreatment with PTX. Similar results were also reported by Hosoi *et al*. (*22*), although a discrepancy was found regarding the EC_{50} for 5-oxo-ETE (about 1 nM in our study compared to about 10 nM in theirs). In addition, they reported an apparent chemokinetic response, while we did not detect significant chemokinetic behavior. Although the reason for these discrepancies remain to be clarified, essentially the same results by the two groups provide direct evidence that the activation of hGPCR48 by its ligand causes the chemotaxis of the receptor-expressing cells toward the ligand.

Several lines of evidence are consistent with the idea that the chemotaxis of granulocytes toward 5-oxo-ETE is mediated through the activation of hGPCR48: (i) Both granulocytes and hGPCR48-expressing CHO cells show chemotactic activities for 5-oxo-ETE in a PTX-sensitive manner; (ii) messenger RNAs for hGPCR48 are expressed in granulocytes; and (iii) the chemotaxis of granulocytes toward 5-oxo-ETE is suppressed by anti-hGPCR48 antibodies. The EC_{50} for 5-oxo-ETE-induced chemotaxis of neutrophils is reported to be 90 nM (*2*), which is 10 to 100 times higher than the values for chemotaxis of hGPCR48-expressing CHO cells and for hGPCR-dependent changes in second messengers. In our experiments, we found an EC_{50} value of 180 nM for the for 5-oxo-ETE–induced chemotaxis of grauloctyes, but this value was variable among granuloctye preparations and was sometimes higher than 180 nM. The discrepancy in the EC_{50} values between granulocytes and hGPCR48-expressing CHO cells suggests the possibility that another GPCR with a lower affinity for 5-oxo-ETE is involved in granulocyte chemotaxis. In general, studies using antagonists are useful, but specific antagonists for hGPCR48 are not yet available. Therefore, we examined the effect of antibodies specific for hGPCR48, and found that granulocyte chemotaxis was partially but significantly inhibited by treatment with the antibody. This effect is not likely to be a nonspecific inhibition of cell migration because the chemotaxis of granulocytes toward LTB_4 was hardly affected by the same treatment. Thus, our results provide strong evidence that the chemotaxis of granulocytes toward 5-oxo-ETE is mediated by hGPCR48, although it is still possible that the chemotaxis is mediated through a GPCR with the same antigenicity as hGPCR48 (that is a GPCR with the same or similar sequence in the first outer loop as hGPCR48). The question remains as to why a higher concentration of 5-oxo-ETE is required for the chemotaxis of granulocytes as compared with the concentrations required for the chemotaxis of CHO cells or changes in second messengers. One possible explanation is that the expression level of hGPCR48 in granulocytes is lower as compared to that in CHO cells. It should be noted that our granulocyte preparations consisted mostly of neutrophils, and the level of the mRNA for hGPCR48 is reported to be much lower in neutrophils than in eosinophils. Another possible explanation is that the downstream signaling of hGPCR48 differs between granuloctyes and CHO cells, and between chemotaxis and changes in second messengers.

We have examined the possibility of the involvement of G proteins other than $G_{i/o}$ in the chemotaxis of CHO cells expressing hGPCR48. In order to examine the contribution of each G protein to a cellular response, it is useful to express inhibitory proteins or peptides specific for each G protein. One tool for this strategy is the carboxyl terminal region of the G protein alpha subunit, which competitively inhibits the receptor–G protein interaction, since the C-terminal region plays a critical role in the interaction with receptors (*19*). Another method is to overexpress the specific RGS domain of G protein–coupled receptor kinases or guanine nucleotide exchange factors (20). We employed carboxyl terminal peptides of $G\alpha_{12/13}$ subunits or the $G_{12/13}$-specific RGS domain of p115RhoGEF to inhibit $G_{12/13}$ activity in the cells. By suppressing the $G_{12/13}$ pathways using these constructs, the number of cells migrating toward 5-oxo-ETE was markedly increased. These results suggest that the downstream signal of G_i is negatively regulated via $G_{12/13}$. It has been reported that the $S1P_3$ receptor mediates the dual regulation of chemotaxis toward S1P with G_i and $G_{12/13}$ (*17*, *18*). Counteracting signals from the G_i and $G_{12/13}$ pathways might act at the same time to regulate chemotaxis toward 5-oxo-ETE as well as chemotaxis toward $S1P_3$. It would be interesting to observe the effect of G12-ct and G13-ct on the chemotaxis of human granulocytes. Unfortunately human granulocytes are too fragile to survive until G12-ct and G13-ct begins to be expressed a few days after transfection. Thus direct evidence remains to be provided for the involvement of $G_{12/13}$ in the hGPCR48-mediated chemotaxis of human granulocytes.

In summary, we provide evidence that hGPCR48 is responsible for the chemotaxis toward 5-oxo-ETE of receptor-expressing CHO cells and human granulocites, and that the chemotaxis mediated by this receptor is simulated by a $G_{i/o}$ pathway and inihibited by a $G_{12/13}$ pathway. It remains to be clarified how the signaling pathways initiated by the activation of hGPCR48 converge through the activation of G_i and $G_{12/13}$ into positive and negative regulation of cell migration.

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