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TG1019/OXE, a $G\alpha_{i/o}$-protein-coupled receptor, mediates 5-oxo-eicosatetraenoic acid-induced chemotaxis

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Abstract

We have previously identified a $G\alpha_{i/o}$-protein-coupled receptor (TG1019/OXE) using 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-oxo-ETE) as its ligand. We investigated signal transduction from TG1019 following stimulation with 5-oxo-ETE and role of TG1019 in 5-oxo-ETE-induced chemotaxis, using Chinese hamster ovary cells expressing TG1019 (CHO/TG1019 cells). 5-Oxo-ETE induced intracellular calcium mobilization and rapid activation of MEK/ERK and PI3K/Akt pathways in CHO/TG1019 cells. CHO/TG1019 cells stimulated with 5-oxo-ETE and other eicosanoids exhibited chemotaxis with efficacies related to agonistic activity of each eicosanoid for TG1019. Pretreatment of the cells with pertussis toxin, a phospholipase C (PLC) inhibitor (U73122) or a PI3K inhibitor (LY294002), markedly suppressed 5-oxo-ETE-induced chemotaxis, whereas pretreatment with a MEK inhibitor (PD98059) had no significant effect on the chemotaxis. Our results show that TG1019 mediates 5-oxo-ETE-induced chemotaxis and that signals from TG1019 are transduced via $G\alpha_{i/o}$ protein to PLC/calcium mobilization, MEK/ERK, and PI3K/Akt, among which PLC and PI3K would play important roles in the chemotaxis.

Keywords: 5-Oxo-ETE; TG1019; OXE; Chemotaxis; PI3K; Phospholipase C; ERK; Calcium mobilization; U73122; LY294002; GPCR; Lipid mediator

Various human inflammatory cells, such as polymorphonuclear leukocytes [1,2], lymphocytes [3], neutrophils [4,5], eosinophils [6], monocytes [3,7], dendritic cells [5], and macrophages [8,9], produce 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-oxo-ETE), an arachidonic acid metabolite, via the 5-lipoxygenase pathway when exposed to oxidative stress, calcium ionophores, or PKC stimulators [10]. 5-Oxo-ETE is a potent chemotactic agent for neutrophils [5,11], monocytes [12], and eosinophils [6,13–16], which are most sensitive to 5-oxo-ETE stimulation. In eosinophils, 5-oxo-ETE activates biological steps involved in cell adhesion, e.g., actin polymerization, L-selectin shedding, and CD11b expression, following rapid calcium mobilization and MAPK activation [11,13,17–19]. In vivo experiments have shown that inhalation of 5-oxo-ETE elicits eosinophil migration into lung tissues in Brown Norway rats [20] and that its subcutaneous injection in rabbit subcutis causes severe edema with eosinophil infiltration into the urticarial lesion [10]. Moreover, intradermal administration of 5-oxo-ETE in humans has been shown to induce infiltration of eosinophils and neutrophils into the skin [21]. Biologically active 5-oxo-ETE was detected in the scales shed by psoriatic patients [10], and in patients with severe pulmonary hypertension [22]. It has been suggested that 5-oxo-ETE might be a pathological mediator of inflammation [10,20–22].

We have previously cloned and characterized a novel $G\alpha_{i/o}$-protein-coupled receptor (TG1019) that is specifically activated by 5-oxo-ETE [23]. Other groups also reported the same GPCR as R527 [24] and hGPCR48 [25], respectively. This GPCR was recently termed as

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OXE by IUPHAR Nomenclature Committee [26]. Using Chinese hamster ovary (CHO) cells transiently expressing TG1019, we have shown that 5-oxo-ETE substantially inhibits forskolin-stimulated cAMP accumulation and that this inhibition is completely abolished by pretreatment with pertussis toxin (PTX) [23]. This finding suggests that TG1019 is coupled with PTX-sensitive $G\alpha_{i/o}$ protein. O'Flaherty et al. inferred that stimulation of neutrophils by 5-oxo-ETE is mediated via a mechanism that involves PTX-sensitive $G\alpha_i$ protein [19,27]. However, it remains unclear whether TG1019 mediates 5-oxo-ETE-induced chemotaxis and how its signal is transduced in this mediation.

Using CHO cells stably expressing TG1019 (CHO/TG1019 cells), we investigated in this study the role of TG1019 in 5-oxo-ETE-induced chemotaxis and clarified TG1019 transduction in these cells following stimulation with 5-oxo-ETE.

Materials and methods

Materials. All eicosanoids used in this study were purchased from Cayman Chemical (Ann Arbor, MI). CHO cells were obtained from American Type Culture Collection (Rockville, MD). Mouse anti-phospho-p44/p42 MAPK E10 monoclonal antibody, rabbit anti-phospho-Akt (Ser473) antibody, anti-Akt polyclonal antibody, the PI3K inhibitor LY294002, and the MEK inhibitor PD98059 were from Cell Signaling Technology (Beverly, MA). The phospholipase C (PLC) inhibitor U73122, its inactive analog (U73343), PTX, lipid-free BSA, and fetal bovine serum were from Sigma (St. Louis, MO). The Rho kinase inhibitor Y27632 was from Calbiochem (San Diego, CA). Rabbit anti-ERK polyclonal antibody was from Santa Cruz (Santa Cruz, CA). HRP-conjugated second antibody and SuperSignal West Pico chemiluminescent substrate were from Pierce (Rockford, IL). DMEM/F12 and RPMI 1640 media were from Invitrogen (Carlsbad, CA). Hygromycin B and bovine fibronectin were from Wako Chemical (Japan).

Construction of CHO cells stably expressing TG1019. An expression vector, pcDNA3.1-TG1019, carrying a full-length cDNA encoding TG1019 was constructed as described previously [23]. CHO cells were transfected with pcDNA3.1-TG1019 or pcDNA3.1 using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. After incubation at 37 °C for 24 h, the cells were reseeded and cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum and 800 μg of hygromycin B per milliliter. Hygromycin B-resistant cells were then cloned and their functional response was evaluated using calcium mobilization assay as described below. The hygromycin B-resistant cells were maintained in the complete medium supplemented with 800 μg of hygromycin B per milliliter.

Calcium mobilization assay. Cells were harvested with 1 mM EDTA in PBS, washed twice with PBS, loaded with 3 μM Fura-2 AM (Dojin, Japan) in an assay buffer containing 0.05% cremophore, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.18% glucose, and 10 mM Hepes (pH 7.4), and incubated at 37 °C for 60 min in a CO_2 incubator. After washing twice with the assay buffer without cremophore, the cells were suspended in the same buffer at a density of 3.7×10^6 cells per milliliter. The cell suspension (80 μl) was then poured into a well of a 96-well plate (3614, Costar), and the reaction was started by adding 20 μl of the ligand solution (5-oxo-ETE, 5(*S*)-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5(*S*)-HPETE), 5(±)-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5(±)-HETE), 5(*S*)-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5(*S*)-HETE), 5(*R*)-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5(*R*)-HETE), 5-oxo-6*Z*, 9*S*-(*S*-glutathionyl)-11*Z*,14*Z*-eicosatrienoic acid (FOG9), or (±)5(6)-epoxy-8*Z*,11*Z*,14*Z*-eicosatrienoic acid (5,6-EET)). In some experiments, the Fura-2 loaded cells were incubated at 37 °C for 10 min with the inhibitors prior to addition of the ligand solution (5-oxo-ETE). Intracellular calcium concentration ($[Ca^{2+}]_i$) was monitored by measuring the ratio (F_{340nm}/F_{380nm}) of fluorescence intensity at 510 nm emitted by excitation with 340 and 380 nm using FDSS6000 (Hamamatsu Photonix, Japan). The fluorescence ratio of Fura-2 saturated with Ca^{2+} was obtained after lysing the Fura-2 loaded cells with 0.5% Triton X-100, whereas the ratio of Ca^{2+}-free Fura-2 was obtained after exposing the lysed cells to 10 mM EGTA. A dissociation constant of 224 nM for Fura-2–Ca^{2+} complex was used to calculate $[Ca^{2+}]_i$ [12].

Western blot analysis. Cells (1.5×10^5 cells) were seeded into a well of a 6-well plate in DMEM/F12 medium supplemented with 10% fetal bovine serum and incubated at 37 °C for 24 h in a CO_2 incubator. The cells were then rinsed twice with serum-free DMEM/F12 supplemented with 0.1% lipid-free BSA and incubated in the serum-free medium at 37 °C for 20 h in a CO_2 incubator. 5-Oxo-ETE was added to the cells and the cells were further incubated at 37 °C for 5–30 min in a CO_2 incubator. In some experiments, the cells were treated for 45 min with the kinase inhibitors prior to the addition of 5-oxo-ETE. After incubation, the supernatant was removed and 200 μl of lysis buffer consisting of 62.5 mM Tris/HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol (DTT), and 0.01% bromophenol blue was added to the well. The cells were then harvested by scraping, sonicated for 30 s, and heated for 5 min at 100 °C. Fifty-five microliters of the cell lysate was electrophoresed on a 11/14 narrow range gradient SDS–PAGE gel (Daiichi Kagaku, Japan) and blotted on a polyvinylidene difluoride (PVDF) membrane (Millipore; Bedford, MA). The membrane was incubated in BlockAce (Dainihon Seiyaku, Japan) for 2 h and then treated with the first antibody in TTBS containing 0.1% Tween 20, 137 mM NaCl, and 20 mM Tris (pH 7.6) at 4 °C for 18 h. After washing with TTBS, the membrane was incubated with HRP-conjugated appropriate second antibody in TTBS at room temperature for 1 h. Proteins were detected with SuperSignal West Pico Chemiluminescent substrate.

Chemotaxis assay. The chemotaxis assay was carried out as described previously [28]. Polycarbonate filters (polyvinylpyrrolidone-free, 8 μm pores, Neuroprobe; Gaithersburg, MD) were coated with 10 μg of bovine fibronectin per milliliter of PBS at room temperature for 30 min and dried. To the lower well of a 96-well microchemotaxis chamber (Neuroprobe) was added 33 μl of ligand solution. The well was then covered with a polycarbonate filter and the upper part of the chamber was fixed in place. Semi-confluent cells were collected with cell-dissociation buffer (enzyme-free PBS-based) (Invitrogen), washed twice with RPMI 1640 (without phenol red) supplemented with 0.1% lipid-free BSA, and suspended in this medium. The suspended cells (3×10^5 cells) were then added to the upper well of the 96-well microchemotaxis chamber and incubated at 37 °C for 2 h in a CO_2 incubator. In order to assess chemokinetic activity, the same amount of ligand added to the lower well of the microchemotaxis chamber was added to the upper well. In some experiments, the cells were treated with the kinase or PLC inhibitor for 20 min prior to the beginning of chemotaxis. The cells on the filter were fixed with methanol, and stained using a Diff-Quik staining Kit (International Reagents, Japan). The cells on the upper side of the filter were wiped off and the number of cells that migrated to the lower side of the filter was determined by measuring absorbance at 600 nm using a 96-well microplate reader (Molecular Devices; Sunnyvale, CA).

Quantitative determination of 5-oxo-ETE. The amount of 5-oxo-ETE having remained after incubation with CHO/TG1019 cells or contaminated in other eicosanoids used in this study was determined by LC/MS spectrometry. All eicosanoids were incubated with the cells at the same density as used in the chemotaxis assay