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G_o is required for the release of IL-8 and TNF-α, but not degranulation in human mast cells

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ABSTRACT

Mast cells activated by IgE-dependent and -independent mechanisms play important roles in innate and acquired immune responses. Activation of pertussis toxin (PTX)-sensitive $G_{i/o}$ proteins is the key step in mast cell degranulation and release of de novo synthesized inflammatory mediators through IgE-independent mechanism. However, the roles of G_i and G_o proteins in mast cells activation have not yet been differentiated. In the current study, the functional roles of G_o proteins in the activities of LAD2 cells, a human mast cell line, are identified. Knockdown of $G\alpha_o$ expression significantly inhibited the synthesis of IL-8 and TNF-α from substance P activated LAD2 cells but demonstrated no effect on degranulation. This effect was associated with the activation of Erk and JNK/MAPKs signaling, whereas PI3K-Akt, calcium mobilization and NFAT translocation remained unchanged. These results suggest that G_i and G_o proteins differentially regulate human mast cells activities through activating distinct signaling cascades.

1. Introduction

Mast cells are tissue resident immune effector cells which play a pivotal role in both innate and adaptive immune responses through the release of preformed biogenic amines and de novo synthesized mediators such as arachidonic acid metabolites, and various cytokines/chemokines. Mast cells can be activated by IgE-dependent and -independent mechanisms. The classical IgE-dependent mechanism involves the binding of antigens to the receptor-bound IgE which leads to the crosslinking of the high-affinity receptors for IgE (FcεRI). For poly-basic secretagogues, such as substance P, somatostatin, vasoactive intestinal peptide (VIP), and antimicrobial peptides, they can either directly activate pertussis toxin (PTX)-sensitive $G_{i/o}$ proteins in mast cells or through activation of G protein-coupled receptors (GPCRs). GPCRs are by far the most abundant type of cell surface receptors and the most common targets for pharmacotherapy (Okayama et al., 2008; Pundir and Kulka, 2010). Over 50% of pharmaceutical agents in use target GPCRs or their signaling molecules (Druey, 2009). A better understanding of their intracellular signaling mechanisms is of critical importance in the development of new drugs for the treatment of mast cells associated diseases.

GPCRs are characterized by their seven transmembrane α-helical regions and their association with the heterotrimeric GTP-binding proteins (G proteins) which consist of α, β and γ subunits. Based on the sequence homology and functional similarities of the α subunits, G protein is divided into four classes: G_s, $G_{i/o}$, G_q and $G_{12/13}$ (He et al., 2006). PTX catalyzes the ADP-ribosylation of the α subunits of the $G_{i/o}$ protein and prevents the G protein heterotrimer from interacting with the receptors, thus blocking their coupling and activation (Mangmool and Kurose, 2011). In contrast, benzalkonium chloride (BAC) differentially inhibits the activation

Abbreviations: Akt, Protein kinase B; BAC, Benzalkonium chloride; FcεRI, High-affinity receptor for IgE; G protein, Guanine nucleotide-binding protein; GPCRs, Gprotein-coupled receptors; JNK, c-Jun N-terminal kinase; LAD2 cells, Laboratory of allergic disease 2 human mast cells; PLC, Phospholipase C; PTX, Pertussis toxin

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of G_i, but potentiates G_o signaling (Gotow and Nishi, 2007). $G_{i/o}$ has long been commonly referred together because it is difficult to distinguish the downstream signaling pathways between G_i and G_o in most cells type (He et al., 2006).

G_o is one of the most abundant proteins in brain tissue and plays critical roles in brain development and functions (Nakamura et al., 2013). However, the pathophysiological roles of G_o protein outside the nervous system have not been thoroughly investigated. Recent studies had demonstrated that aberrant G_o signaling or mutation of GNAO1 (gene name for $G\alpha_o$) promotes oncogenesis in several cancer types (Bratton et al., 2012; Garcia-Marcos et al., 2011; Kan et al., 2010), but the roles of G_o signaling in immune system remain undefined. Our previous study has demonstrated that substance P-induced degranulation and release of IL-8 from the human mast cell line LAD2 cells were both blocked by PTX, whereas BAC differentially inhibited degranulation but potentiated IL-8 release (Yu et al., 2013). These results strongly indicate that G_i and G_o could differentially regulate the activities of human mast cells. We thus hypothesize that G_o protein contributes only to the release of de novo synthesis of cytokines while G_i protein regulates both degranulation and synthesis of cytokines in mast cells. The present study is designed to test this hypothesis and to characterize the associated signaling mechanisms. While activation of $G_{i/o}$ protein has been shown to induce mast cell degranulation through the phosphorylation of PLCβ and the subsequent activation of protein kinase C (PKC) and calcium mobilization (Kajiwara et al., 2010), the synthesis of cytokines are believed to involve MAPKs (Erk, JNK, p38), PI3K, NF-κB and Ca^{2+}/calcineurin/NFAT (Azzolina et al., 2003; Okabe et al., 2000; Zaidi et al., 2006). We thus focus our studies on these signaling cascades.

2. Materials and methods

2.1. Culture of human mast cells

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA) (Kirshenbaum et al., 2003). Cells were maintained in StemPro-34 medium supplemented with 10 ml/l StemPro nutrient supplement, 1:100 penicillin-streptomycin, 2 mmol/l L-glutamine, 100 ng/ml human stem cell factor, and 50 ng/ml interleukin-6 in an atmosphere containing 5% CO_2 at 37 °C. Culture medium was replaced every 2 weeks and the cells were kept at a density of 2×10^5 cells/ml.

2.2. Chemical Reagents

Substance P was purchased from Sigma (St Louis, MO). Antibodies against NFAT, NF-κB, total and phosphorylated Erk, p38, JNK and Akt were purchased from Cell Signaling (Danvers, MA). Antibodies against alpha-tubulin and Lamin B1 were purchased from Protein Tech (Chicago, IL). Antibodies against $G\alpha_o$ protein was from Merck Millipore (Lake Placid, NY).

2.3. Lentivirus-mediated Knockdown of GNAO1 in Human Mast Cell

GNAO1-targeted shRNA lentivirus and a scrambled control nontarget lentivirus were purchased from Genechem (Montreal, Quebec). The shRNA that gave the highest knockdown efficiency (24124-1) was used. Cell transduction was conducted by mixing 1 ml of viral supernatant with 1 ml of LAD2 cells (1×10^6 cells). Medium was changed to virus-free complete medium 8 h post-infection. Puromycin (2 μg/ml) was added to select cells with stable virus integration into the genome after a recovery period of 24 h. Cells were analyzed for GNAO1 knockdown after two weeks of antibiotic selection.

2.4. RNA extraction and qRT-PCR

Total RNA was isolated with TRIzol reagent and was quantified by measuring the ratio of $A_{260\ nm}/A_{280\ nm}$. RNA (1 μg) was then reverse-transcribed with the Revert Aid First Strand cDNA Synthesis Kit (Thermo scientific, Waltham, Massachusetts, USA). The RT mixture was incubated for 50 min at 42 °C followed by 15 min at 70 °C. Quantitative PCR was performed using the SYBR Green Dye method which was carried out using cDNAs supplemented with SYBR Green supermix (Bio-rad, Hercules, California, USA) and 100 nM paired primers for different genes to be analyzed in PCR buffer. The PCR protocol consisted of a cycle at 95 °C for 5 min followed by 40 cycles consisting of 15 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C. The $2^{-\Delta\Delta Ct}$ method was used for quantification of the target gene expression. All tests were done in triplicates. The average Ct was calculated for the target genes and the internal control (GAPDH) and the ΔCt ($Ct_{,target}$-$Ct_{,GAPDH}$) values were then determined.

2.5. Degranulation assay

β-hexosaminidase (β-hex) is an enzyme contained in the cytoplasmic granules of mast cells and the level of this enzyme released into the supernatant provides an indication of the degree of degranulation process immediately occurs following mast cell activation. LAD2 cells were first incubated with substance P for 30 min and the levels of β-hex in supernatants and cell lysates were determined by a colorimetric assay in which release of p-nitrophenol from 4-nitrophenyl N-acetyl-β-D-glucosaminide was measured (Kasakura et al., 2009). The absorbance was measured at 405 nm with 605 nm taken as a reference by using a multiplate reader. The level of β-hex released into the supernatant was calculated as the percentage of the total β-hex content. All results were corrected for spontaneous β-hex release in buffer alone and were less than 5%.

2.6. IL-8 and TNF-α measurement

LAD2 cells were incubated with substance P for 24 h to allow synthesis and release of IL-8 and TNF-α. The amounts of IL-8 and TNF-α in the supernatants were measured by ELISA assay (BD Biosciences) according to the manufacturer's instructions. All results were corrected for spontaneous IL-8 and TNF-α release that was less than 22 pg/10^6 cells.

2.7. Intracellular Ca^{2+} mobilization assay

LAD2 cells were loaded with 2 μM Fluo-3AM (Invitrogen) for 30 min at 37 °C. The cells were washed three times and then resuspended in HEPES buffer with human albumin prior to challenging with different stimuli. Fluo-3-loaded mast cells were viewed with a laser scanning confocal microscope. The images were captured at every 10 s intervals. Fluorescence images were obtained at wavelengths of 506 nm with an emission wavelength of 526 nm. The overall levels of calcium influx were compared with area under the curve analysis. F_x/F_0 was the ratio of fluorescence of time point X (F_x) divided by that of time point zero (F_0).

2.8. Western blotting

Following stimulation, cells were washed twice with cold phosphate buffered saline and lysed in lysis buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 10 mM