Prostaglandins, Leukotrienes and Essential Fatty Acids 105 (2016) 1–8

Contents lists available at ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

journal homepage: www.elsevier.com/locate/plefa

Cellular signalling of cysteinyl leukotriene type 1 receptor variants $CysLT_1$-G300S and $CysLT_1$-I206S

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ARTICLE INFO

Article history:
Received 14 May 2015
Received in revised form
5 November 2015
Accepted 12 December 2015

Keywords:
Cysteinyl-leukotriene
$CysLT_1$
Variant
Signalling
Atopy

ABSTRACT

Cysteinyl-leukotrienes are pro-inflammatory lipid mediators, involved in allergic asthma, that bind the G-protein-coupled receptors $CysLT_1$, $CysLT_2$ and GPR99. A polymorphism in one of these receptors, $CysLT_1$-G300S was strongly associated with atopy, whereas the $CysLT_1$-I206S polymorphism was not. In the present work, our aim was to characterize these two variants by studying their cellular signalling. Cell surface expression of mutant receptors in transfected HEK-293 cells was comparable to that of the wild-type receptor. Compared to $CysLT_1$-WT, production of inositol phosphates as well as IL-8 and IL-13 promoter transactivation in response to either LTD_4 or LTC_4 was significantly increased in $CysLT_1$-G300S-transfected cells. Moreover, LTD_4-induced phosphorylation of the signalling effector Erk, but not p38, p65 or c-Jun was higher in $CysLT_1$-G300S-transfected cells. On the other hand, the variant $CysLT_1$-I206S did not show a significant difference in its signal transduction compared to the wild-type receptor. Taken together, our results indicate that the variant $CysLT_1$-G300S can induce a greater signal than the $CysLT_1$-WT receptor, a feature that may be relevant to its association with atopy.

1. Introduction

The cysteinyl-leukotrienes (cysLTs), LTC_4, LTD_4, and LTE_4, are pro-inflammatory lipid mediators, involved in many pathologies including allergic asthma and rhinitis. They are derived from arachidonic acid and synthesized through the 5-lipoxygenase pathway in different cell types, including leukocytes (mast cells, basophils, eosinophils and macrophages…), platelets and endothelial cells [1].

CysLTs bind to at least three G-protein-coupled receptors (GPCRs). $CysLT_1$ and $CysLT_2$, share 38% homology in structure. $CysLT_1$ receptor binds preferentially LTD_4 ($LTD_4 > LTC_4 \gg LTE_4$), whereas the $CysLT_2$ receptor has no preference between LTD_4 and LTC_4 ($LTC_4 = LTD_4 \gg LTE_4$) [2,3]. A third receptor, GPR99, has been recently characterized and shown to be an LTE_4 receptor, with less affinity for the other two cysLTs ($LTE_4 \gg LTD_4 > LTC_4$) [4].

The $CysLT_1$ receptor is mostly expressed in human lung, on smooth muscle cells and macrophages, but also on peripheral blood cells, such as eosinophils, basophils, monocytes and B and T lymphocytes [5–7]. On the other hand, the $CysLT_2$ receptor is more ubiquitous and is found on interstitial lung macrophages and on circulating eosinophils and monocytes [8]. These two receptors are also expressed in other organs, like on the spleen, intestines, pancreas and prostate for $CysLT_1$ receptor, and in the heart, brain, adrenal gland, placenta and spleen for $CysLT_2$ receptor [9–11].

The $CysLT_1$ receptor is highly expressed in the lung, which may be relevant for its implication in pulmonary diseases like asthma. In fact, specific antagonists of the $CysLT_1$ receptor (pranlukast, montelukast and zafirlukast) are used in the clinic to control bronchoconstriction and inflammation in asthma and allergic rhinitis [12].

In the midst of an inflammatory environment, $CysLT_1$ receptor expression is up-regulated by many cytokines, including the Th2 cytokines IL-4 and IL-13 in monocytes and macrophages, IL-5 in eosinophils, IL-1β in HUVEC, TGFβ, IFN-γ and IL-13 in pulmonary smooth muscle cells [7,13–19].

As a GPCR, the $CysLT_1$ receptor is coupled to the G-proteins Gαi and Gαq [20,21]. Through different pathways, including MAPKinases and NF-κB, it induces the transcription of several genes implicated in inflammation, such as IL-8, MCP-1, IL-4, TGFβ, P-selectin, CXC chemokine ligand 2 and NO synthase [22–27].

Polymorphisms in the $CysLT_1$ receptor have been identified which are associated with atopy, an important risk factor for asthma. In the English and Spanish populations, the variant T927C, due to a synonymous mutation in the $CysLT_1$ receptor gene was

Abbreviations: cysLTs, cysteinyl-leukotrienes; CysLT1, cysteinyl-leukotriene type 1 receptor; WT, wild-type; LT, leukotriene; GPCR, G-protein-coupled receptor

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http://dx.doi.org/10.1016/j.plefa.2015.12.004
0952-3278/

associated to atopy [28]. Atopy is a genetic predisposition to develop allergies, characterized by an elevation of IgE, and is often associated with the production of inflammatory mediators such as cysLTs [29]. In addition, a serine substitution in the amino acid structure of the $CysLT_1$ receptor resulted in the variants $CysLT_1$-G300S and $CysLT_1$-I206S, the only identified variants of $CysLT_1$ due to exonic mis-sense mutations. Those have been discovered and studied in the genetically isolated population of Tristan Da Cunha, which has a high prevalence of atopy and asthma. The receptor variant $CysLT_1$-G300S has been significantly associated with atopy whereas the polymorphism $CysLT_1$-I206S was not associated with atopy, in this population [30,31].

We hypothesized that the atopic phenotype may be partially influenced by an altered binding and/or signalling due to the amino-acid substitution in the receptor protein. The principal aim of this study was to investigate the signalling mechanisms of the variants $CysLT_1$-G300S and $CysLT_1$-I206S induced by their natural ligands, LTD_4 and LTC_4, in comparison with the CysLT1-WT receptor.

2. Materials and methods

2.1. Materials

LTC_4 and LTD_4 were obtained from Cayman Chemical (Ann Arbor, MI, USA). [^{3}H]LTD_4 was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA, USA). The IP-One HTRF assay kit was from CisBio Bioassays (Bedford, MA, USA). Specific antibodies against p-p65 Ser536, Erk1/2, p-p38 Thr180/Tyr182, and p-c-Jun Ser65 were from Cell Signaling Technology (Danvers, MA, USA), and antibodies against p65, p-Erk, p38, and Jun were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The anti-cMyc antibody was from mouse hybridoma (9E10), from American Type Culture Collection (Manassas, VA, USA), and Cy5-conjugated goat anti-mouse antibody was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Aprotinin, 4-(2-aminoethyl)-benzenesulfonyl fluoride, leupeptin, NaF, soybean trypsin inhibitor, Na_3VO_4, and fetal bovine serum were from Sigma-Aldrich (Oakville, QC, Canada). TransIT-LT1 was purchased from Mirus Bio LLC (Madison, WI, USA), and Dulbecco's modified Eagle's medium (DMEM; high glucose) was from Invitrogen, (Burlington, ON, Canada).

2.2. Plasmids

The $CysLT_1$ constructs bearing the 899G > A and 617T > G nucleotide substitutions that encode the G300S and I206S variants were kind gifts from Dr. Valérie Capra (U. of Milan, Milan, Italy) [30]. They were in the mammalian expression vestor pcDNA3 (Invitrogen, Carlsbad, CA). Since our wild-type $CysLT_1$ construct was also in pcDNA3 (pcDNA3-cmyc-$CysLT_1$-WT), but with a β-globin intron fragment for stability and a cMyc tag for labelling [24], we extracted a 1043 bp fragment from, pcDNA3-cmyc-$CysLT_1$-WT using unique NdeI and BbvC1 restriction sites, which contained the intron and the cMyc tag, and inserted it into the variant constructs, thus yielding the cMyc-labelled pcDNA3-cmyc-$CysLT_1$-G300S and pcDNA3-cmyc-$CysLT_1$-I206S.

The human IL-8 promoter luciferase construct hIL8/luc (−162/+44) and IL-13 promoter luciferase construct hIL13/luc (−940/+48) were kind gifts from Dr. Allan R. Brasier (University of Texas Medical Branch, Galveston, TX, USA) and Dr. Lisa Cameron (University of Alberta, AB, Canada), respectively.

2.3. Cells

HEK-293 cells (American Type Culture Collection) were cultured in DMEM with high glucose, supplemented with 5% fetal bovine serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). Transient transfections were carried out with TransIT-LT1, and experiments were performed 48 h after transfection. Although the transfections are transient, the cells will be named $CysLT_1$-WT, $CysLT_1$-G300S and $CysLT_1$-I206S.

2.4. Surface expression of the receptors

Cells were transfected with cDNAs coding for $CysLT_1$-WT, $CysLT_1$-G300S and $CysLT_1$-I206S receptors tagged on the N-terminal end with the epitope Myc. 48 h after transfection, cells were washed with Phosphate Buffer Saline (PBS) twice, harvested then fixed with 2% paraformaldehyde for 15 min at room temperature. After that, cells were washed with PBS again then blocked in Bovine Serum Albumine (BSA) 2% for 30 min at room temperature. Anti-cMyc antibody was added to the cells at a concentration of 1 mg for 10^6 cells, for 1 h of incubation. After three washes with PBS, the secondary anti-mouse antibody was added for 1 h and again washed three times. Cells were analyzed by a dual-laser FACSCalibur cytometer (Becton Dickinson, Mountain View, CA, USA).

2.5. Inositol phosphate production assay

After 48h of transfection, cells ($CysLT_1$-WT, $CysLT_1$-G300S and $CysLT_1$-I206S) were harvested, and 20 000 cells were plated in a 384 well white plate, and the experiment was conducted as per the CisBio IP-one assay kit's manufacturer's instructions. In brief, cells were suspended in a buffer (10 mM Hepes, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl, pH 7.4), then stimulated for 30 min with LTC_4 or LTD_4 from 0.01 to 100 nM. Exogenous IP1 coupled to a dye d2, is added to the cells, then, monoclonal antibody specific to IP1 labelled with lumi4-tb cryptate which competes with the native IP1 produced by the cells. After 1 h of incubation, fluorescence was measured at 665 and 620 nm, using fluorescence plate reader TECAN M1000 (Männdorf, Switzerland). The intensity of the FRET signal obtained is inversely proportional to the quantity of IP1 produced by the cells.

2.6. Luciferase assays

HEK-293 cells were plated in 24-well tissue culture plates for 24 h. The cells were then transfected with 20 ng of cDNA of the receptors $CysLT_1$-WT, $CysLT_1$-G300S or $CysLT_1$-I206S, 0.05ng of *IL8/luc* or empty vector, per well, using 0.75 μl of TransIT-LT1 transfection reagent. After 24 h, cells were serum-starved overnight before stimulation with LTC_4, LTD_4, or Ethanol (EtOH) for 6 h. Cell lysates were assayed for luciferase activity using the Dual-Glo luciferase system (Promega, Madison, WI, USA).

2.7. Western blotting

In brief, the transfected cells $CysLT_1$-WT, $CysLT_1$-G300S and $CysLT_1$-I206S, plated in six-well plates (serum starved over-night) were stimulated with LTC_4, LTD_4 or EtOH for the indicated times and concentrations. After appropriate time, cells were lysed in buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 5% sodium deoxycholate, 10% TritonX-100, 10 mM NaF, 100 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 1 mM Na_3VO_4, 1nM Calyculin A, and 15 nM okadaic acid) for 30 min on ice. Lysates were separated on an 8% SDS-