International Immunopharmacology 49 (2017) 6–12

Contents lists available at ScienceDirect

International Immunopharmacology

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

LL-37-induced human mast cell activation through G protein-coupled receptor MrgX2

Yangyang Yu[a], Yuanyuan Zhang[d], Yarui Zhang[c], Yihong Lai[a], Wenwen Chen[a], Zhangang Xiao[e], Wei Zhang[c], Meiling Jin[a,*,1], Bo Yu[b,**,1]

[a] Center for Diabetes, Obesity and Metabolism, Department of Physiology, Shenzhen University Health Science Center, Shenzhen, Guangdong, China
[b] Department of Dermatology, Peking University Shenzhen Hospital, Shenzhen, Guangdong, China
[c] Shenzhen Key Laboratory for Translational Medicine of Dermatology, Biomedical Research Institute, Shenzhen Peking University-The Hong Kong University of Science and Technology Medical Center, Guangdong, China
[d] Central Laboratory & Center of Prenatal Diagnosis, Shenzhen Baoan Maternal and Child Health Hospital, Baoan District, Shenzhen, Guangdong, China
[e] Laboratory of Molecular Pharmacology, Department of Pharmacology, School of Pharmacy, Southwest Medical University, Luzhou, 646000, Sichuan, PR China

ARTICLE INFO

Keywords:
Mrgx2
G protein
LL-37
Human mast cell

ABSTRACT

Human LL-37 is an important class of cationic antimicrobial peptide (CAP) that is known to stimulate mast cell activation. While many studies have been conducted on LL-37, to date little is known about the functional receptors for LL-37-induced human mast cell activation, in particular in terms of the release of de novo synthesized mediators. Thus, the aim of the present study is to identify the functional receptors for LL-37-induced human mast cell activation in terms of the degranulation and release of de novo synthesized mediators and investigate the downstream signalling pathways involved in mast cell activation. Overall, our study importantly demonstrates that LL-37-induced human mast cell degranulation and release of de novo synthesized mediators function primarily through the activation of MrgX2. We furthermore show that LL-37-induced human mast cell line LAD2 cells are involved in the degranulation and release of IL-8, and that FPRL1 and P2X7 have only a partial effect on IL-8 release, and no effect on mast cell degranulation triggered by LL-37. Instead, we find that silencing the expression of MrgX2 in human mast cell significantly inhibits the LL-37-induced degranulation and release of IL-8. Overall, this effect is associated with the activation of the G_i protein, PLC/PKC/Calcium/NFAT, PI3K/Akt and MAPKs signalling pathways.

1. Introduction

Antimicrobial peptides (AMPs), such as defensins and cathelicidins are produced by activated epithelial cells, platelets and myeloid cells, and are important innate immune factors for the host defence system [1]. Amongst all varieties of cathelicidins, peptide 18 (hCAP18), also known as LL-37, is the only human-related cathelicidin identified to date. Recent studies have importantly demonstrated that LL-37 acts as an antimicrobial peptide against Gram-negative and Gram-positive bacteria, alongside functioning as an active immune modulator [2]. Indeed, LL-37 has been implicated in the pathogenesis of many inflammatory diseases, such as rosacea and urticaria [3,4]. Further to this, it has been shown that LL-37 can induce the activation of immune effector cells, such as monocyte, microglia and mast cell through secretion of various inflammatory cytokines and chemokines including, IL-6, IL-8 and MCP-1 [2,3,5]. Importantly, LL-37 can also induce mast cell degranulation and chemotaxis [6,7].

Previous studies have revealed that LL-37's actions are mediated by the activation of G protein-coupled receptors (GPCRs), such as formyl peptide receptor 2 (FPR-2 or FPRL1) on neutrophils and T cells [8] and purinergic receptor P2X7 on neutrophils and macrophages [8,9]. Further studies have shown that LL-37-induced mast cell migration can be significantly inhibited by pertussis toxin (PTX), however, competitive binding assays revealed that FPRL1 (FPR-2) was unlikely

Abbreviations: Akt, protein kinase B; AMPs, antimicrobial peptides; BAC, benzalkonium chloride; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; FPR-2 or FPRL1, formyl peptide receptor 2; G protein, guanine nucleotide-binding protein; GPCRs, G protein-coupled receptors; InsP3, inositol trisphosphates; JNK, c-Jun *N*-terminal kinase; LAD2 cells, laboratory of allergic disease 2 human mast cells; Mrgs, Mas-related genes; NFAT, nuclear factor of activated T-cells; PI3K, phosphatidylinositol-3 kinase; PKC, protein kinase C; PLC, phospholipase C; PTX, pertussis toxin

* Correspondence to: M. Jin, School of Medicine, Shenzhen University, Shenzhen, Guangdong 518060, China.
** Correspondence to: B. Yu, Department of Dermatology, Peking University Shenzhen Hospital, Shenzhen, Guangdong 518036, China.
E-mail addresses: y102513@163.com (M. Jin), yubomd@163.com (B. Yu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.intimp.2017.05.016
Received 9 February 2017; Received in revised form 10 May 2017; Accepted 16 May 2017
Available online 23 May 2017

to be a functional receptor for LL-37 in rat mast cells [6]. On the other hand, studies have also indicated that mast cells also express ionotropic receptors which directly contribute to Ca^{2+} signalling and improvement of mast cell degranulation [10]. To this end, it has been suggested that LL-37 could interact with the ionotropic receptor P2X7 and induced the release of IL-8 and IL-1β via ERK signalling in human gingival fibroblast [11]. Furthermore, the activation of P2X7 by ATP in mast cells also induces the release of inflammatory cytokines, chemokines and leukotrienes which contribute to the recruitment of neutrophils [12]. A key challenge, in this respect, is to ascertain the roles of FPRL and P2X7 in LL-37-induced human mast cells activity, which to date remain unclear.

Recently, the novel GPCR Mas-related gene (Mrg), was identified in mast cells, and was found to be primarily expressed on the neurons of dorsal root ganglia and trigeminal ganglia [13]. The Mrg gene family member, MrgX2, was recently shown to be involved in the activation of human mast cells via interactions with basic peptides, such as LL-37, human-β-defensin (HBD) and substance P [14,15]. Further to this, it was reported that silencing the expression of MrgX2 by specific MrgX2-shRNA results in significant inhibition of LL-37-induced Ca^{2+} mobilization and degranulation in human mast cell [16]. Furthermore, studies show that activation of MrgX2 in keratinocyte partially induces the release of IL-6 via ERK and NF-κB signalling cascades [17]. In this context, although MrgX2 has been shown to induce human mast cell degranulation, the role of MrgX2 in the LL-37-induced human mast cell release of de novo synthesized mediators is yet to be determined.

While there is some evidence to suggest that cationic host defence peptides interact directly with mast cells via G protein-coupled receptors and ionotropic receptors, very little is known about the downstream signalling involved in LL-37-induced mast cell activation. In mast cell activation signalling cascades, migration and degranulation require the activation of PLC/PKC/calcium mobilization signalling pathways [18], while the release of de novo synthesized mediators involves the activation of mitogen activated protein kinases (MAPKs, including extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK)), phosphatidylinositol-3 kinase (PI3K), NF-κB and calcium/calcinuerin/NAFT pathways [19,20]. With all this in mind, our overall goal in the present study is to firstly, identify the functional receptors for LL-37-induced human mast cell activation with respect to the degranulation and release of de novo synthesized mediators, and thereafter, to ascertain the downstream signalling pathways involved in this mast cell activation.

2. Materials and methods

2.1. Human mast cell culture

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). Cells were maintained in a StemPro-34 medium supplemented with 10 ml/l StemPro nutrient supplement, 1100 penicillin-streptomycin, 2 mmol/l L-glutamine, 100 ng/ml human stem cell factor and 50 ng/ml interleukin-6 in a 5% CO_2 environment maintained at 37 °C. The 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

LL-37 was purchased from GL Biochem; Pam3CSK4 was purchased from Invivogen; SB203580, ciclosporin A, U73122, Boc-MLF and Bay 11-7821 were obtained from Tocris; Peptidoglycan (PGN) from *S. aureus*, Ro31-8220 and KN-62 were purchased from Sigma; Wortmannin was obtained from Cayman; and PD98059, SB 203580 and JNK Inhibitor II were sourced from Calbiochem. The final concentration of DMSO did not affect the normal response of LAD2 cells when chemicals were dissolved in DMSO.

2.3. Lentivirus-mediated knockdown of MrgX2 in human mast cells

MrgX2-targeted shRNA lentivirus and a scrambled controlled non-target lentivirus were purchased from Shanghai Genechem. Cell transduction was conducted by mixing 1 ml of viral supernatant with 1 ml of LAD2 (1×10^6 cells). The medium was changed to a 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. The cells were analysed for MrgX2 knockdown after 2 weeks of antibiotic selection.

2.4. Degranulation assay

β-Hexosaminidase (β-hex) is an enzyme contained in the cytoplasmic granules of mast cells and the degree of release of this enzyme into the supernatant provides an indication of the degranulation process following mast cell activation. LAD2 cells were respectively incubated with different stimuli for 30 min and β-hex release was measured. The β-hex in supernatants and cell lysates was determined by use of a colorimetric assay in which release of *p*-nitrophenol from 4-nitrophenyl *N*-acetyl-β-D-glucosaminide was measured [21]. The absorbance was measured at 405 nm using a multiplate reader with the reading at 605 nm utilised as the reference. The percentage of β-hex release was calculated as the percentage of the total β-hex content. All results were corrected for a spontaneous β-hex release of < 5%.

2.5. IL-8 measurement

LAD2 cells were pre-incubated with various inhibitors for the corresponding time periods prior to incubation with different stimuli for 24 h to facilitate the synthesis and release of IL-8. The release of IL-8 in the supernatants was measured using an ELISA assay (BD Biosciences) according to the manufacturer's instructions. All results were corrected for a spontaneous IL-8 release of < 22 pg/10^6 cells.

2.6. Western blotting

The whole cell lysate of the LAD2 cells was conducted using a RIPA buffer, and the nuclear and cytoplasmic proteins were subsequently extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Sangon, China). The Western blotting was conducted as previously reported (17). The primary antibodies were prepared as follows: anti-Akt (cell signaling, Beverly, MA); anti-phospho-Akt (Ser 473, cell signaling, Beverly, MA); anti-JNK (cell signaling, Beverly, MA); anti-phospho-JNK (Thr 183/Tyr 185, cell signaling, Beverly, MA); anti-ERK (cell signaling, Beverly, MA); anti-phospho-ERK (Thr 202/Tyr 204, cell signaling, Beverly, MA); anti-p38 MAPK (cell signaling, Beverly, MA); anti-phospho-p38 MAPK (Thr 180/Thr 182, cell signaling, Beverly, MA); anti-NFAT (cell signaling, Beverly, MA); anti-NF-κB (cell signaling, Beverly, MA); anti-LaminB (Abcam, USA); anti-β-tubulin (ProteinTech, China). A secondary antibody (1:1000 diluted) (MBL) was subsequently added to the membrane for 1 h at room temperature. After incubation in an ECL solution (Pierce Chemical, Rockford, IL), the protein levels were detected and analysed using a LAS-1000 Pro image reader.

2.7. RNA extraction and qRT-PCR

Cell pellets were collected by centrifugation and were then lysed with TRIzol reagent to extract the RNA. The total RNA was isolated using TRIzol reagent and was quantified by measuring the ratio of A260nm/A280nm. RNA (1 μg) was then reverse transcribed using a Revert Aid First Strand cDNA Synthesis Kit (Thermo scientific, Waltham, Massachusetts, USA). The mixture was incubated for 50 min at 42 °C and then 15 min at 70 °C. qPCR was performed using the SYBR Green Dye method which was carried out using cDNA