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LAT is essential for the mast cell stabilising effect of tHGA in IgE-mediated mast cell activation

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ABSTRACT

Mast cells play a central role in the pathogenesis of allergic reaction. Activation of mast cells by antigens is strictly dependent on the influx of extracellular calcium that involves a complex interaction between signalling molecules located within the cells. We have previously reported that tHGA, an active compound originally isolated from a local shrub known as *Melicope ptelefolia*, prevented IgE-mediated mast cell activation and passive systemic anaphylaxis by suppressing the release of interleukin-4 (IL-4) and tumour necrosis factor (TNF)-α from activated rat basophilic leukaemia (RBL)-2H3 cells. However, the mechanism of action (MOA) as well as the molecular target underlying the mast cell stabilising effect of tHGA has not been previously investigated. In this study, DNP-IgE-sensitised RBL-2H3 cells were pre-treated with tHGA before challenged with DNP-BSA. To dissect the MOA of tHGA in IgE-mediated mast cell activation, the effect of tHGA on the transcription of IL-4 and TNF-α mRNA was determined using Real Time-Polymerase Chain Reaction (qPCR) followed by Calcium Influx Assay to confirm the involvement of calcium in the activation of mast cells. The protein lysates were analysed by using Western Blot to determine the effect of tHGA on various important signalling molecules in the LAT-PLCγ-MAPK and PI3K-NFκB pathways. In order to identify the molecular target of tHGA in IgE-mediated mast cell activation, the LAT and LAT2 genes in RBL-2H3 cells were knocked-down by using RNA interference to establish a LAT/LAT2 competition model. The results showed that tHGA inhibited the transcription of IL-4 and TNF-α as a result of the suppression of calcium influx in activated RBL-2H3 cells. The results from Western Blot revealed that tHGA primarily inhibited the LAT-PLCγ-MAPK pathway with partial inhibition on the PI3K-p65 pathway without affecting Syk. The results from RNAi further demonstrated that tHGA failed to inhibit the release of mediators associated with mast cell degranulation under the LAT/LAT2 competition model in the absence of LAT. Collectively, this study concluded that the molecular target of tHGA could be LAT and may provide a basis for the development of a mast cell stabiliser which targets LAT.

1. Introduction

Allergy is a hypersensitivity disorder mediated by immunological mechanisms which can cause tissue damage and life-threatening reactions, including atopic dermatitis, asthma, and anaphylactic shock [1,2]. One of the key characteristics of allergy is the excessive activation of mast cells by an antigen specific immunoglobulin E (IgE) antibody which results in extreme inflammatory responses [1,3,4]. Mast cells are the major effector cells of allergic inflammation, where their roles in innate and adaptive immune responses have been increasingly recognised within the last few years [5]. The binding of antigen-specific IgE to FcεRI sensitizes mast cells, enabling them to release mediators in response to subsequent encounter with that specific antigen [3,4,6].

The rat basophilic leukaemia cell line (RBL-2H3), which is known to be a mast cell analogue, has been widely used to determine the signal transduction of FcεRI as these cells express abundant FcεRI receptors on their cell surfaces [7–9]. RBL-2H3 cells release preformed and newly synthesised mediators of immune allergic response following crosslinking of IgE-bound FcεRI with multivalent allergens [7,10–12]. In addition, activated RBL-2H3 cells produce various cytokines, chemokines, prostaglandins and leukotrienes that play important roles in the infiltration of

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inflammatory cells and the induction of the late-phase reactions [4,13]. The effect of these mediators on surrounding cells and tissues is what causes the symptoms and severity of an allergic reaction [6].

Signalling pathways leading to the degranulation of mast cells after engagement of the IgE on the FcεRI receptor have been well studied and extensively characterised [14–16]. Following FcεRI aggregation, a complex series of intracellular signalling processes within mast cells will be initiated [17]. Although the immediate receptor-proximal signalling events seem to be common for the release of all categories of mediators, the receptor-distal signalling events must diverge to regulate the different mechanisms by which these mediators are released [15]. Several lines of evidence have shown that the LAT-PLCγ axis as well as the PI3K-NFκB axis pathways play vital roles in coordinating the IgE-dependant mast cell degranulation [15,18,19]. Both pathways are activated by a tyrosine kinase known as Spleen tyrosine kinase (Syk) through the phosphorylation of adapter molecules such as linker for activation of T cells (LAT) and Non-T cell activation linker (LAT2) [20]. This will lead to the downstream cascade effects which cause the activation of various key enzymes or molecules along the signalling pathways such as phosphoinositide 3-kinase (PI3K), NFκB p65, phospholipase Cγ (PLCγ), mitogen-activated protein kinase (MAPK) and arachidonic acid-associated enzymes [14,15]. Eventually, this will result in the massive influx of Ca^{2+} from the surrounding environment into the cell which is considered an important event that triggers mast cell degranulation [20,21].

As mast cell degranulation has been shown to play an important role in allergic inflammation, coagulation cascades, host defence, and tissue remodelling, increased understanding of mast cell degranulation and the mechanisms involved will lead to the discovery of effective therapy for diseases associated with mast cell degranulation [22]. Until now, the treatment of allergic diseases relies on clinically-prescribed drug classes of mast cell stabilisers or H_1 antagonists such as ketotifen fumarate, olopatadine and cromolyn [6]. These mast cell stabilisers have been reported to attenuate the release of allergic mediators such as histamine, leukotrienes C_4 and PAF, as well as playing a part in the signalling pathway that is associated with Ca^{2+} influx inhibition during mast cell activation [23]. For example, cromolyn has been reported to inhibit NF-κB nuclear translocation and the action of phosphorylated MAPK proteins during mast cell activation [24,25]. At the same time, unwanted side effects such as drowsiness, upset stomach, chest congestion and dry mouth have been associated with the use of these mast cell stabilisers [26]. As such, there are ongoing researches into potential mast cell stabilising agents derived from natural sources, however their mechanisms of action and safety are yet to be established [6].

2,4,6-Trihydroxy-3-geranylacetophenone (tHGA) is an active compound originated from a local shrub, namely *Melicope ptelefolia* [27]. It has been traditionally used by locals to treat a wide range of diseases, including itches [28]. Scientific evidence on the anti-allergic properties of tHGA has been previously reported where the oral and systemic treatment of tHGA prevented the ovalbumin-induced allergic airway inflammation in a murine model of allergic asthma [29,30]. Despite the important roles of mast cells as the main immune effector cells involved in the pathogenesis of allergic diseases, there was no report on the mast cell stabilising effect of tHGA until recently, when our research group demonstrated that tHGA inhibited IgE-mediated mast cell degranulation and passive systemic anaphylaxis [31]. However, the mechanism underlying its protective effects is still poorly understood. Therefore, the purpose of this study was to investigate the underlying mechanism of the inhibitory effects of tHGA in a cellular model of IgE-mediated mast cell degranulation.

2. Material and methods

2.1. Compound synthesis and preparation

tHGA, with its chemical structure previously reported [30], was synthesised according to a previously described method [30] and the stock solution (20 mM) was prepared according to a previous study [31]. Prior to experiments, the activity of several batches of synthetic tHGA was tested *in vitro* using the β-hexosaminidase release assay. There was minimal variation between different batches of tHGA (<3% variation).

2.2. Antibodies and reagents

Mouse anti-dinitrophenol (DNP) monoclonal IgE and 4-Nitrophenyl N-acetyl-β-D-glucosaminide (PNAG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DNP-conjugated bovine serum albumin (DNP-BSA) was purchased from Calbiochem (San Diego, CA, USA). The enzyme-linked immunoassay (EIA) kits for histamine, prostaglandins D_2 (PGD_2) and leukotrienes C_4 (LTC_4) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The ELISA kits for interleukin 4 (IL-4) and tumour necrosis factor-alpha (TNF-α) were purchased from R&D systems (Minneapolis, MN, USA). Ketotifen fumarate was purchased from MP Biomedicals (Santa Ana, California, USA). Eagle's minimum essential medium (EMEM), foetal bovine serum, penicillin and streptomycin were purchased from Life Technologies Inc. (Waltham, MA, USA). Silencer® Select siRNAs (LAT, LAT2, GAPDH and negative control), Opti-MEM® I-reduced serum media and Lipofectamine® RNAiMAX transfection reagent were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). FLUOFORTE® Calcium assay kit was purchased from Enzo Life Sciences (Farmingdale, NY, USA). The QuantiNova™ SYBR® Green PCR kit and RNeasy® Plus Mini kit were purchased from QIAGEN Inc. (Valencia, CA, USA). Rabbit polyclonal primary antibodies against 5-LOX, COX-2, IKK, PI3K, PLCγ1, p38, JNK, ERK, p65, Syk, LAT, β-Actin, TFIIB, phospho-Syk, phospho-PLCγ1, phospho-p38, phospho-JNK, phospho-ERK, phospho-IKK, phospho-PI3K, phospho-LAT, HRP-conjugated goat anti-rabbit IgG secondary antibodies, HRP-conjugated mouse monoclonal IgG_1 for β-actin and mouse monoclonal IgG_{2b} for TFIIB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal primary antibodies against IκB, $cPLA_2$, phospho-IκB and phospho-$cPLA_2$ were purchased from Cell Signalling Technology (Danvers, MA, USA). SuperSignal West Pico Chemiluminescent Substrate was purchased from Pierce Biotechnology (Rockford, IL, USA). Other Western Blotting chemical reagents were purchased from Amresco LLC (Solon, OH, USA). ADP-Glo™ Kinase assay and Syk Kinase Enzyme System assay kits were purchased from Promega Corporation (Madison, WI, USA).

2.3. Cell culture and activation

RBL-2H3 cell line (rat basophilic leukaemia cell line) was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in EMEM medium containing 10% foetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. RBL-2H3 cells were incubated at 37 °C in a 5% CO_2 humidified incubator and were subcultured to new T25 tissue culture flask (3×10^5 cells/flask) or used for assays when the confluence of cells reached 80%. Only RBL-2H3 cells of passage number ranging from 6 to 11 were used throughout this study.