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A steroid-mimicking nanomaterial that mediates inhibition of human lung mast cell responses

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Abstract

Water-soluble fullerenes can be engineered to regulate activation of mast cells (MC) and control MC-driven diseases *in vivo*. To further understand their anti-inflammatory mechanisms a C_{70}-based fullerene conjugated to four *myo*-inositol molecules (C_{70}-I) was examined *in vitro* for its effects on the signaling pathways leading to mediator release from human lung MC. The C_{70}-I fullerene stabilizes MC and acts synergistically with long-acting β_2-adrenergic receptor agonists (LABA) to enhance inhibition of MC mediator release through FcεRI-simulation. The inhibition was paralleled by the upregulation of dual-specificity phosphatase one (DUSP1) gene and protein levels. Concomitantly, increases in MAPK were blunted in C_{70}-I treated cells. The increase in DUSP1 expression was due to the ability of C_{70}-I to prevent the ubiquitination and degradation of DUSP1. These findings identify a mechanism of how fullerenes inhibit inflammatory mediator release from MC and suggest they could potentially be an alternative therapy for steroid resistant asthmatics.

Key words: Fullerenes; Inhaled corticosteroids; Mast cell; Dual-specificity phosphatase one

1. Introduction

The use of corticosteroids (also known as glucocorticosteroids, glucocorticoids, or steroids) is the hallmark treatment for patients suffering from many chronic inflammatory and immune diseases (such as asthma) yet the treatment response is extremely variable among patients. Specific subsets of patients are non-responsive to such treatments and are termed steroid resistant. These patients often fail to respond to even extremely high dosages of inhaled corticosteroids (ICS), therefore much research has been focused on understanding the mechanisms of ICS action to better understand non-responsive patients.[1] Several different studies have found that the dual-specificity phosphatase 1 (DUSP1) is a prospective regulator of this ICS response.[2–6] Dual-specificity phosphatase 1 is one in a family of protein phosphatases which has been shown *in vitro* to inactivate mitogen-activated protein kinases (MAPK).[7] Upregulation of DUSP1 directly results in the de-phosphorylation/inactivation of mitogen-activated protein kinase (MAPK) via dephosphorylation of the threonine and/or tyrosine residues and thus acts as a regulator through a negative feedback mechanism.[8–10] The induction of the DUSP1 gene and subsequent inhibition of MAPKs result in the reduced expression of pro-inflammatory cytokines[11–13]

While ICS therapy remains a successful first line treatment for persistent cases of asthma, consistent use of steroids can have many harmful side effects. In addition, polymorphisms in DUSP1 expression are associated with clinical effectiveness of ICS therapy for asthma.[5] For patients nonresponsive to ICS therapy β_2-adrenergic receptor agonists are used. However, the link between DUSP1 expression and patient responses to ICS/β_2-adrenergic receptor agonists is not completely understood. Many cell types respond to steroids by upregulating DUSP1, which dephosphorylates and inactivates both MAPK and JNK resulting in significant reductions in mediator release and the production of pro-inflammatory cytokines.

Lung mast cells (MC) are effector cells in the asthmatic response[14] and release of their asthma-triggering mediators has been shown to be inhibited by steroids[15,16] and β_2-adrenergic

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receptor agonists.[17] The role of DUSP1 expression in activated human lung MC following pharmacological interventions has not been studied. Clues for a role for DUSP1 in MC-driven responses came from studies where MC from knockout mice lacking DUSP1 show enhanced degranulation and are highly susceptible to anaphylaxis.[18] Mast cell stabilizing nanomaterials were also shown to increase DUSP1 gene expression which paralleled inhibition of mediator release.[19] Based upon these similarities, we investigated the mechanism underlying C_{70}-Tetrainositol (C_{70}-I) inhibition of lung MC mediator release and compare its effects to those of steroids and β_2-adrenergic receptor agonists. As shown below C_{70}-I inhibited both FcεRI-mediated degranulation and GM-CSF cytokine production which was paralleled with increases in DUSP1 expression. Furthermore, C_{70}-I synergized with long-acting β_2-adrenergic receptor agonists (LABA) to potentiate this inhibition. These findings provide mechanistic insight into how C_{70}-I can mediate MC degranulation and cytokine production and how DUSP1 polymorphisms could influence varying responses in patient ICS treatment[5] through the upregulation of lung MC DUSP1 levels.

2. Methods

2.1. C_{70}-I synthesis and characterization

The C_{70}-I was synthesized by conjugating four *myo*-inositol molecules (*cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol) to each C_{70} carbon cage via Bingel–Hirsch cyclopropanation reaction.[20,21,22] C_{70}-I has two inositol moieties at each of the two poles of the oval-shaped C_{70} molecule. These *myo*-inositol moieties solubilize the C_{70} molecule in aqueous media. *Myo*-inositol has been shown to significantly reduce inflammation in two widely used animal models for inflammation.[23] The final compound was characterized using matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), nuclear magnetic resonance (^{1}H and ^{13}C NMR), high-performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FTIR), and dynamic light scatter/zeta potential (DLS/ζ). C_{70}-I was found to be approximately 30 nm in diameter and fully dispersed in buffered saline, it has a molecular formula of $C_{100}H_{44}O_{32}$ with MW of 1756 Da, and a zeta potential of −16.90 mV (Supplemental Figure 1A). Additionally, cell toxicity was assessed by incubation with concentrations shown effective for inhibition (10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M) and viability counts taken on days three, six, and nine. No toxicity was observed using trypan blue exclusions compared to control cells (Supplemental Figure 1B). Pre-incubation of MC with a fluorophore tagged-C_{70}-I (Texas Red) revealed uptake of fullerenes by MC with high efficiency at concentrations down to 10^{-10} M after five hours of incubation (Supplemental Figure 1C).

2.2. Gene microarray

Mast cells were harvested from tissue received by the Cooperative Human Tissue Network and cultured using methods previously described.[24–26] Mast cells (>95%; 1×10^7 cell/condition; each condition performed in triplicate) were incubated with or without C_{70}-I (10^{-5} M for 16 h; 37 °C; 6% CO_2), washed in fresh warm (37 °C) media and supernatants removed (to remove pre-formed mediators), and fresh warm medium alone (negative) or medium containing anti-FcεRI Abs (1 μg/ml) added for two hours at 37 °C. Cells were centrifuged, the supernatant and the pellet immediately frozen and microarray performed using the Human Whole Genome OneArray™ gene expression profiling service (Phalanx Biotech Group) report. Microarray analysis was performed and analyzed as described previously.[19] The UNCG Human Studies International Review Board approved all studies.

2.3. Immuno-blotting and immuno-precipitation

Mast cells (~3.0×10^6 cells/ml) were pretreated with or without C_{70}-I and either cross-linked with anti-FcεRI Abs (0.5 μg/ml) or left untreated in a 37 °C incubator with 6% CO_2. Cells were lysed using methods previously described.[24–26] For immuno-precipitations (IP), lysates were pre-cleared with Protein A/G agarose beads, followed by overnight incubation with anti-DUSP1 (Abcam, Cambridge, MA) conjugated beads at 4 °C and washed extensively to isolate DUSP1 proteins. Precipitates were subjected to SDS-PAGE (10% SDS gels; Life Technologies, Grand Island, NY) and immuno-blotting (IB) as described.[24] For highly efficient and quantitative exposure, IR_{700} or IR_{800}-conjugated secondary antibodies were measured using Licor's Odyssey infrared imaging system (Licor Inc. Lincoln, NE). The band intensities were quantified as described[27] and normalized to the house-keeping gene, β-actin.

2.4. Mast cell mediator release

Lung MC of the MC_T (tryptase-positive, chymase-negative; predominant type seen in the alveoli of the lung >93%)[28] were incubated for 16 h with or without C_{70}-I and ICS alone (at increasing dosages to evaluate dose response), or in combination with β_2-adrenergic receptor agonists or both overnight (16 h; 37 °C; 6% CO_2).[29] Cells were washed and activated with optimal concentrations (0.5 μg/ml) of anti-FcεRI Abs (3B4) for two hours (β-Hex) or 16 h (cytokines) at 37 °C. Mediator release was measured as described previously.[25,30] Incubation times and concentrations were previously optimized and it was determined that 70-carbon fullerenes were taken up by MC through an endocytosis-dependent mechanism and persisted in the MC for up to one week, predominantly homing to the ER, but also to a lesser degree found in the mitochondria and lysosomes.[29]

3. Results

3.1. Pretreatment of MC with C_{70}-I upregulates DUSP1 gene and protein levels

Microarray analysis was used to obtain a broad overview of those FcεRI-associated signaling molecules influenced by C_{70}-I pre-incubation following FcεRI activation.[19] Interestingly, C_{70}-I caused a dramatic increase in expression of wide range of genes, including several within the DUSP family. Notably, DUSP1 increased relative gene expression levels approximately 55%