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The anti-inflammatory and pro-resolution effects of aspirin-triggered RvD1 (AT-RvD1) on peripheral blood mononuclear cells from patients with severe asthma

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ABSTRACT

Asthma is an inflammatory disease that is characterized by a predominance of eosinophils and/or neutrophils in the airways. In the resolution of inflammation, lipid mediators such as resolvin D1 (RvD1) and its epimer aspirin-triggered RvD1 (AT-RvD1) are produced and demonstrate anti-inflammatory and pro-resolution effects. In experimental models such as airway allergic inflammation induced by ovalbumin in mice, RvD1 and AT-RvD1 alleviate some of the most important phenotypes of asthma. Here, we demonstrated the effects of AT-RvD1 on peripheral blood mononuclear cells (PBMCs) from healthy individuals and patients with severe asthma stimulated with lipopolysaccharide (LPS) or *Dermatophagoides pteronyssinus* (DM). AT-RvD1 (100 nM) reduced the concentration of TNF-α in PBMCs from healthy individuals and patients with severe asthma stimulated with LPS or DM. In addition, AT-RvD1 lowered the production of IL-10 only in PBMCs from patients with severe asthma stimulated with LPS. These effects were associated in part with decreasing NF-κB activation. Moreover, AT-RvD1 significantly increased phagocytosis of apoptotic neutrophils by monocytes from patients with severe asthma. In conclusion, AT-RvD1 demonstrated both anti-inflammatory and pro-resolution effects in PBMCs from patients with severe asthma and could become in the future an alternative treatment for asthma.

1. Introduction

Asthma is an inflammatory disease of the airways characterized by migration and accumulation of leukocytes (particularly eosinophils), mucus hypersecretion, increased production of immunoglobulin E (IgE) and bronchial hyperreactivity [1]. The asthma guidelines classify the disease severity as mild, moderate, or severe [2]. The pathophysiology of allergic asthma is mediated by $CD4^+$ T cell immune responses. Although knowledge of the roles of different T cell subsets in asthma has increased in recent years, the Th2-type immune responses are most classically associated with the pathology of asthma [3]. Th2 cytokines such as IL-4, IL-5 and IL-13 are involved in asthma but beyond these, other cytokines such as TNF-α (pro-inflammatory) and IL-10 (anti-inflammatory) also play significant roles in the modulation of airway inflammation [4–6]. Asthma exacerbations are common, and may be triggered by a number of atmospheric and domiciliary environmental factors such as allergens, bacteria and viruses [7]. The prevention and treatment of asthma exacerbation are the most important clinical outcomes for optimal asthmatic healthy individuals [8]. Most patients with asthma have symptoms that are readily controllable by standard asthma therapies. However, 5–10% of asthmatic individuals have a poor response to the traditional treatment, leading to exacerbations or symptoms that are refractory to current therapy [9]. During inflammation, the essential omega (Ω)-3 fatty acid, docosahexaenoic acid (DHA; C22:6), is available for enzymatic transformation into several anti-inflammatory and pro-resolving mediators, including the D-series resolvins [10]. The resolvins demonstrate anti-inflammatory effects as well as pro-resolution effects, such as activation of macrophage

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phagocytosis [11–12]. Aspirin acetylates cyclooxygenase-2 (COX-2) leading to the formation of aspirin-triggered mediators, including the 17 R epimer of RvD1 termed aspirin-triggered RvD1 (AT-RvD1), which is more resistant to catalysis than RvD1 [13,14]. Two specific human GPCRs, ALX/FPR2 and GPR32, mediate the action of RvD1s [15]. ALX/FPR2 is broadly expressed in many cells, including monocytes/macrophages, neutrophils, immature dendritic cells, T cells/B cells, endothelial cells, epithelial cells, fibroblasts and others [2,4–7,16]. GPR32 is expressed in macrophages [11] and polymorphonuclear leukocytes [17,18]. Our group has demonstrated AT-RvD1 anti-inflammatory and/or pro-resolving effects in several experimental models, including acute lung injury induced by acid [19], experimental airway allergic inflammation induced by ovalbumin in mice [20] and bronchial epithelial cells stimulated with IL-4 [21]. Peripheral blood mononuclear cells (PBMCs) of asthmatics respond to key factors such as allergens and LPS. PBMCs are easily observable and modulated by systemic inflammation in chronic diseases such as asthma. Therefore, in this study we evaluated the effects of AT-RvD1 on PBMCs from patients with severe asthma stimulated with LPS or DM, as well as the phagocytic activity of monocytes to apoptotic neutrophils.

2. Materials and methods

2.1. Patients

These studies were approved by the Universidade Federal do Triangulo Mineiro Committee for the Ethics of Human Research (number 2343). Subjects with severe asthma were recruited using criteria defined by the Severe Asthma Research Program [22]. Non-asthmatic and healthy individuals were selected based on the absence of a history of asthma or clinical symptoms and a negative skin prick test. The exclusion criteria were the use of antihistamines in the week before the skin test and previous or current immunotherapy, or autoimmune or infectious disease.

2.2. Skin prick test

All individuals underwent skin prick tests with the following commercial allergen extracts: dust mite (*Blomia tropicalis*, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), extract of cockroach (*Blattella germanica* and *Periplaneta americana*), mold (*Alternaria alternata*) and pet dander (*Felis domesticus* and *Canis familiaris*) obtained from Anthygenus (Brazil). A mean wheal diameter 3 mm or larger than the negative healthy individuals (diluent) was considered to be positive. Patients with a positive skin prick test to *D. pteronyssinus* were selected for blood collection.

2.3. Sample collection

After the skin prick test, peripheral blood was collected by venipuncture from healthy subjects ($n = 6$) and severe asthmatics ($n = 10$) in a heparinized tube. From each patient, 10–20 mL of venous blood was collected. The PBMCs were isolated by density-gradient centrifugation over Histopaque 1077 (Sigma Aldrich). RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin was used as the complete culture medium for further analysis.

2.4. Stimulus and treatment

The AT-RvD1 was donated by Dr. David Bruce Levy from Brigham and Women's Hospital. PBMCs (1×10^6 cell/mL) were cultivated in 96-well plates and were treated with AT-RvD1 (100 nM) or vehicle (ethanol 0.01%) 30 min before the LPS (1 μg/mL) [23] or DM (10 μg/mL) [24] stimulation.

2.5. TNF-α and IL-10 production

The supernatant was collected 24 h after stimulation, and TNF-α and IL-10 concentrations were determined by ELISA according to the manufacturer's instructions (R & D Systems, or BD Pharmingen).

2.6. NF-κB pathways

The effect of AT-RvD1 on NF-κB pathways was assessed by cytometry according to [25]. Briefly, 15 min after LPS or DM stimulation, cells were fixed with pre-warmed BD Cytofix Buffer (4% paraformaldehyde) for 10 min at 37 °C. After centrifugation, the cells were permeabilized in ice-cold methanol for 30 min and then stained with mouse monoclonal antibodies to anti-phospho-NF-κB p65 (BD Biosciences Pharmingen, Phosflow, USA; Catalog Number 558421) or their corresponding mouse IgG1 isotype (BD Biosciences Pharmingen, Phosflow, USA) for 60 min followed by FITC- and PE-conjugated goat anti-mouse IgG1 secondary antibody for another 45 min at 10 °C in dark. The cells were then washed, resuspended, and subjected to analysis. The expression of intracellular phosphorylated signaling molecules in 50,000 viable cells was analyzed by flow cytometry (FACS Calibur; BD Biosciences).

2.7. Human monocyte phagocytosis

Human PBMCs and PMNs were isolated using a Ficoll-Histopaque density gradient. PBMCs were plated at a density of ~5×10^6 cells/mL in 8 cm_2 in media (RPMI 1640 plus 10% FCS containing L-glutamine and antibiotics) and incubated 2 h at 37 °C. Non-adherent cells were removed, and fresh monocytes were supplemented before culturing for 5 days in RPMI-1640 medium supplemented with 10% FCS. At day 5, the monocytes were detached with PBS-EDTA, washed, and plated in a 96-well plate (5×10^5/well). PMNs from the healthy blood donors were isolated and resuspended in RPMI-1640 medium supplemented with 10% FCS and labeled with carboxyfluorescein diacetate (10 μM, 30 min at 37 °C) and allowed to undergo apoptosis in RPMI plus 10% FBS (5×106 cells/mL) for 18 h. Monocytes (1×10^5 cells/well) from the healthy individuals or asthmatic patients were incubated with AT-RvD1 (1–100 nM) or vehicle (ethanol 0.01%) for 15 min at 37 °C. Apoptotic neutrophils were added at 1:5 (monocytes:apoptotic neutrophils). Phagocytosis was carried out at 37 °C for 60 min, and fluorescence was monitored with a fluorescence plate reader [11].

2.8. Statistical analysis

The results were expressed as the mean ± standard error of the mean. The evaluation of the results was performed by analysis of variance (ANOVA) followed by a Tukey's post-hoc test. p values <0.05 were considered statistically significant.

3. Results

3.1. AT-RvD1 reduces cytokine production by PBMCs stimulated with LPS

AT-RvD1 has significant anti-inflammatory and pro-resolution effects in experimental airway models such as acute lung injury [25] and allergic airway inflammation induced by ovalbumin in mice [9]. In this study, we evaluated the effects of AT-RvD1 in PBMCs from healthy individuals and subjects with severe asthma stimulated by different agents. In the first set of experiments, LPS was used. LPS induced elevations in TNF-α and IL-10 concentrations in PBMCs from healthy individuals (Fig. 1A & B, respectively) and patients with severe asthma (Fig. 1C & D, respectively) compared to unstimulated cells. AT-RvD1 (100 nM) decreased the TNF-α concentration in PBMCs from healthy individuals when compared to cells stimulated with LPS (Fig. 1A). TNF-α was reduced by ~62%, from 1058.55 ± 212.90 (LPS) to 686.52 ± 19.34 (LPS + AT-RvD1) (mean pg/mL $n = 6$; $p < 0.05$). In addition, AT-RvD1