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AT-RvD1 modulates the activation of bronchial epithelial cells induced by lipopolysaccharide and *Dermatophagoides pteronyssinus*

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ABSTRACT

Bronchial epithelial cells are essential to airways homeostasis; however, they are also involved in exacerbation of airway inflammatory responses of patients with conditions such as asthma. *Dermatophagoides pteronyssinus* (Dp), the most important allergen, and lipopolysaccharide (LPS), both of which are present in house dust mites (HDM), can activate immune and structural cells (such as bronchial epithelial cells) and modulate the airway inflammation in asthma patients. Resolvin D1 (RvD1) and its epimer aspirin-triggered-resolvin D1 (AT-RvD1) are lipid mediators that are produced during the resolution of inflammation and demonstrate anti-inflammatory and pro-resolution effects in several experimental models including experimental models of allergic airway inflammation. Here, we evaluated the effects of AT-RvD1 (10^{-12}–10^{-10} M) on human bronchial epithelial cells (BEAS-2B) stimulated with LPS (2 μg/ml) or Dp (10 μg/ml). After 24 h, the C-C motif chemokine ligand 2 (CCL-2) production was increased in cells that had been stimulated with LPS and Dp compared to the control. However, AT-RvD1 (10^{-11} and 10^{-10} M) significantly reduced the concentration of CCL-2 in a manner that was dependent on the N-formyl peptide receptor 2 (FPR2/ALX) and nuclear factor kappa B (NF-κB) pathways in cells stimulated with LPS or Dp compared to controls. In addition, AT-RvD1 reduced the phosphorylation of signal transducer and activator of transcription (STAT)6 and STAT1 in cells stimulated with Dp and LPS, respectively. In conclusion, AT-RvD1 demonstrated significant anti-inflammatory effects in bronchial epithelial cells that were stimulated with LPS or Dp, which provides new perspectives for therapeutic strategies to control inflammatory airway diseases.

1. Introduction

Bronchial epithelial cells play important roles in the pathophysiology of several airway inflammatory diseases (Hammad and Lambrecht, 2008). These cells express pattern-recognition receptors such as Toll-like receptors (TLR) and protease-activated receptors (PARs), which recognize microorganisms and allergens, respectively (Kauffman, 2006; Kato et al., 2007). The activation of these receptors induces the production of chemokines and cytokines and the expression of adhesion molecules (Ebeling et al., 2007) that can influence dendritic cell maturation and T cell differentiation and consequently modulate the airway inflammation (Reibman et al., 2003; Kiss et al., 2007). Bacteria and their products (such as lipopolysaccharide (LPS)) as well as allergens (such as *Dermatophagoides pteronyssinus* (Dp)) are involved in the initiation as well as the exacerbation of airway inflammation in asthma patients (Möller et al., 2012; Kanchongkittiphon et al., 2014).

An uncontrolled immune response promotes tissue and organ damage. Lipid mediators such as resolvins and their epimers are produced in the resolution of inflammation, and these mediators play significant roles in dampening the inflammatory process (Serhan, 2010). Resolvin D1 (RvD1) and its epimer aspirin-triggered-resolvin D1 (AT-RvD1) are derived from the essential omega-3 fatty acid docosahexaenoic acid (DHA; C22:6). RvD1 and AT-RvD1 demonstrate anti-inflammatory and pro-resolution effects in several experimental models including the ovalbumin-induced allergic airway inflammation model (Rogerio et al., 2012). In addition, AT-RvD1 decreases the production of C-X-C motif chemokine ligand 8 (CXCL8) and C-C motif chemokine ligand 2 (CCL-2) as well as the phosphorylation of nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 6 (STAT6) in bronchial epithelial cells that are stimulated with IL-4 (Oliveira et al., 2015). Here, we extended these results and evaluated the role of AT-RvD1 in bronchial epithelial cells stimulated with LPS or Dp.

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2. Materials and methods

2.1. Cells

Bronchial epithelial cells: The human bronchial epithelial cell line BEAS-2B (ATCC, Rockville, MD) was cultured in Dulbecco's modified Eagle's medium (DMEM-F12/Gibco-Life Technologies, Carlsbad, Calif., USA) supplemented with 10% foetal bovine serum (Gibco-Life Technologies) and 1% penicillin + streptomycin (Gibco-Life Technologies, Carlsbad, Calif., USA) and incubated at 37 °C in a humidified atmosphere with 5% CO_2 and 95% ambient air.

2.2. Stimulus and treatment

AT-RvD1 was donated by Dr. David Bruce Levy of the Harvard Medical School. BEAS-2B (4×10^4 cell/ml) cells were cultured in 96-well plates and treated with AT-RvD1 (10^{-12}–10^{-10} M) or vehicle (0.04% ethanol) for 30 min prior to stimulation with LPS (2 μg/ml) (InvivoGen, Toulouse, France) or Dp (10 μg/ml) (FDA Allergenic, Rio de Janeiro, Brazil). In another set of experiments, we followed the same experimental procedure, except that the cells were previously (15 min before AT-RvD1) treated with BOC1 (10 μM) (MP Biomedical, Solon, France) (Oliveira et al., 2015; Zambalde et al., 2016).

2.3. CCL-2

The supernatant was collected 24 h after LPS or Dp stimulation, and the CCL-2 concentrations were measured by enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (BD Pharmingen, San Diego, Calif., USA).

2.4. NF-κB, STAT1 and STAT6

The effects of AT-RvD1 on the NF-κB, STAT1 and STAT6 pathways were assessed by cytometry according to Cao et al. (2010). Briefly, 24 h after LPS or Dp stimulation, the cells were fixed with pre-warmed BD Cytofix Buffer (4% paraformaldehyde) for 10 min at 37 °C. The cells were then washed and permeabilized in ice-cold Perm/Wash Buffer (BD Biosciences Pharmingen (Phosflow, USA). For the staining with mouse monoclonal antibodies, the cells were incubated with antibodies against phospho-NF-κB (BD Biosciences Pharming- Phosflow, USA), phospho-STAT1 (BD Biosciences Pharmingen- Phosflow, USA), or phospho-STAT6 (BD Biosciences Pharming-Phosphorus, USA) diluted in cold methanol for 60 min at 10 °C in the dark. These cells were then further incubated with PE- or FITC-conjugated goat anti-mouse IgG2b for another 60 min at 10 °C in the dark. The cells were then washed, resuspended and subjected to analysis. The same protocol was carried out with the respective isotype controls. The expression of intracellular phosphorylated signalling molecules in 50,000 viable cells was performed using a FACSCalibur flow cytometer (BD Biosciences).

2.5. Statistical analysis

The results are expressed as the mean ± standard error of the mean. An evaluation of the results was performed using analysis of variance (ANOVA) followed by a Tukey post-test among the means using Graph Pad PRISM (Version 6.0; Graph Pad Software Inc., San Diego, CA, USA). P values less than 0.05 were considered statistically significant.

3. Results

3.1. AT-RvD1 reduces the concentration of the chemokine CCL-2

Our results demonstrated that either LPS or Dp increased the CCL-2 production compared to the control group (Fig. 1A and B, respectively). AT-RvD1 (10^{-11} and 10^{-10} M) significantly decreased the CCL-2 production compared to cells stimulated by LPS (Fig. 1A) or Dp (Fig. 1B). The CCL-2 production was reduced by ~66% from 653.3 ± 78.5 in the cells treated with LPS to 289.6 ± 76.96 (LPS + AT-RvD1 at 10^{-10} M) or 302.7 ± 80.25 (LPS + AT-RvD1 at 10^{-11} M) (mean ± S.E.M.; n =7; $P < 0.05$). In addition, the CCL2 level was reduced by ~46% from 716.6 ± 68.9 in the cells treated with Dp to 386.7 ± 82.2 (Dp + AT-RvD1 at 10^{-10} M) or 386.7 ± 82.2 (Dp + AT-RvD1 at 10^{-11} M). AT-RvD1 (10^{-12} M) did not significantly reduce the CCL2 production. The vehicle did not cause any significant difference in the responses of cells that were stimulated with LPS or Dp (data not shown). AT-RvD1 at 10^{-10} M was chosen for the subsequent experiments.

3.2. The inhibitory effect of AT-RvD1 on CCL2 production is FPR2/ALX receptor-dependent

As described above, LPS as well as Dp increased the CCL2 production, and AT-RvD1 reduced its production (Fig. 2A and B). Interestingly, BOC1, an FPR2/ALX antagonist, prevented the reduction in CCL-2 that was induced by AT-RvD1 in the presence of either LPS or Dp (Fig. 2A and B, respectively).

3.3. AT-RvD1 down-regulates the phosphorylation of NF-κB

LPS and Dp induced the phosphorylation of NF-κB compared to the control group (Fig. 3A and B, respectively). AT-RvD1 (10^{-10} M) significantly decreased the phosphorylation of NF-κB compared to cells that were stimulated with LPS (Fig. 3A) or Dp (Fig. 3B). NF-κB was reduced by ~90% from 0.94 ± 0.22 (LPS) to 0.094 ± 0.032 (LPS + AT-RvD1) (mean ± S.E.M.; n =5; $P < 0.05$) and reduced by ~85% from 1.84 ± 0.95 (Dp) to 0.28 ± 0.14 (Dp + AT-RvD1) (mean ± S.E.M.; n =5–8; $P < 0.05$).

3.4. AT-RvD1 down-regulates the phosphorylation of STAT1 and STAT6

In addition to the NF-κB pathway, the role of AT-RvD1 in the STAT1 and STAT6 activation induced by LPS and Dp, respectively, was also evaluated. LPS increased the phosphorylation of STAT1 and Dp induced phosphorylation of STAT6 compared to control group (Fig. 4A and B, respectively). AT-RvD1 (10^{-10} M) significantly decreased the phosphorylation of STAT1 (Fig. 4A) and STAT-6 (Fig. 4B) compared to cells that were stimulated by LPS or Dp, respectively. STAT1 was reduced by ~46% from 1.55 ± 0.16 (LPS) to 0.84 ± 0.15 (LPS + AT-RvD1) (mean ± S.E.M.; n =5; $P < 0.05$), and STAT6 was reduced by ~45% from 2.45 ± 0.12 (Dp) to 1.37 ± 0.29 (Dp + AT-RvD1) (mean ± S.E.M.; n =4–6; $P < 0.05$).

4. Discussion

In this study, AT-RvD1 demonstrated anti-inflammatory effects as indicated by a reduction in CCL-2 production by bronchial epithelial cells stimulated with LPS or Dp. These effects were dependent on the FPR2/ALX receptor and NF-κB pathways. In addition, AT-RvD1 decreased STAT1 and STAT6 activation induced by LPS and Dp, respectively.

In airway diseases and other inflammatory diseases, the balance between pro-inflammatory and anti-inflammatory mediators is shifted to favour the pro-inflammatory mediators. Resolvins and their epimers are produced during the resolution process and are known to affect the anti-inflammatory and pro-resolution processes (Marion-Letellier et al., 2015). Earlier results from our group have demonstrated that AT-RvD1 plays a significant role in dampening the airway inflammation in several experimental models, including the in vivo ovalbumin-induced experimental allergic airway model (Rogerio et al., 2012) as well as in models of in vitro allergic responses (bronchial epithelial cells