

Allergen Challenge Increases Anandamide in Bronchoalveolar Fluid of Patients With Allergic Asthma

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Preclinical studies suggest that the endocannabinoid anandamide restrains allergic airway obstruction and inflammation. Therefore, we applied saline or allergens via bronchoscopy into different lung segments of patients with allergic asthma. Twenty-four hours later, anandamide concentrations in bronchoalveolar lavage fluids had increased more than fourfold. Anandamide concentrations correlated with severity of airway inflammation. These observations suggest that allergen exposure specifically activates the pulmonary endocannabinoid system (ECS) in patients with allergic asthma.

Bronchial asthma is a chronic inflammatory airway disorder associated with episodic airway obstruction. Approximately 300 million people worldwide are affected, and the prevalence is rising in many populations.¹ Allergic sensitization and allergic rhinitis are risk factors for developing asthma.² Despite several available pharmacologic treatment options, new drug targets that modify airway inflammation and obstruction are needed. The endocannabinoid system (ECS)—consisting of the cannabinoid receptors (CB1 and CB2), expressed on various cell types, and endogenous lipid ligands such as anandamide—is a reasonable target.³ Whereas tobacco smoking can exacerbate asthma, the smoking of marijuana^{4,5} or the inhalation of a Δ^1 -tetrahydrocannabinol-containing aerosol^{6,7} increases airway conductance. The CB1/CB2-agonist CP55,940 has been shown to relieve ovalbumin-induced cough, bronchial constriction, pulmonary mast cell degranulation, and pulmonary inflammation in sensitized guinea pigs.⁸ Inhaled anandamide has been shown to attenuate leukotriene D4-induced airway obstruction in guinea pigs.⁹ Mice lacking CB1, CB2, or both, and those treated with a CB receptor antagonist displayed exacerbated T-cell-mediated

allergic skin inflammation.¹⁰ The inflammatory response was attenuated in animals lacking the anandamide-degrading enzyme fatty acid amide hydrolase (FAAH) and in those treated with CB receptor agonists.¹⁰ These observations suggest that anandamide release could serve as an inhibitory feedback mechanism restraining airway obstruction and inflammation in allergic asthma. We hypothesized that pulmonary anandamide concentration increases with experimental allergen exposure.

RESULTS

We enrolled 15 patients (mean age 39 ± 1.8 years) with a history of mild intermittent allergic asthma and positive airway response to inhaled allergens. Selective segmental saline and allergen challenges and subsequent bronchoalveolar lavages (BALs) using bronchoscopy were performed and were well tolerated by all patients.

Allergic airway disease is invariably associated with pulmonary eosinophilia. BAL eosinophilic granulocyte counts were $0.10 \pm 0.03 \times 10^6$ in saline-stimulated lung segments and $15.8 \pm 5.1 \times 10^6$ in allergen-stimulated lung segments ($P = 0.0001$) (Figure 1a). Interleukin 5 (IL-5), recognized as an eosinophilic granulocyte-colony-stimulating factor at the time of its original discovery,¹¹ is a crucial factor in mediating allergy-associated eosinophilic airway infiltration.¹² IL-5 concentrations in BAL fluid were shown to rise more after allergen challenge as compared with saline challenge (594.3 ± 150.8 pg/ml vs. 54.0 ± 11.3 pg/ml, $P = 0.0001$) (Figure 1b). BAL concentrations of eotaxin, an eosinophil chemotactic factor, were 155.5 ± 31.9 pg/ml after segmental allergen provocation and 71.3 ± 17.4 pg/ml after saline ($P = 0.0017$) (Figure 1c).

We next determined whether experimental airway inflammation is associated with increased airway anandamide

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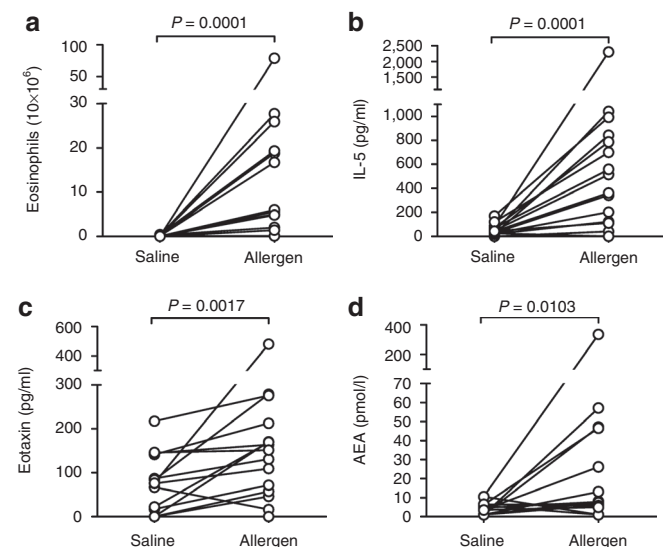


Figure 1 Concentration differences of measured biomarkers in bronchoalveolar lavage (BAL) fluid after saline or allergen challenge. (a) Eosinophilic granulocyte counts, (b) IL-5 concentrations, (c) eotaxin concentrations, and (d) anandamide concentrations in BAL fluid after saline and allergen provocation. AEA, anandamide; IL, interleukin.

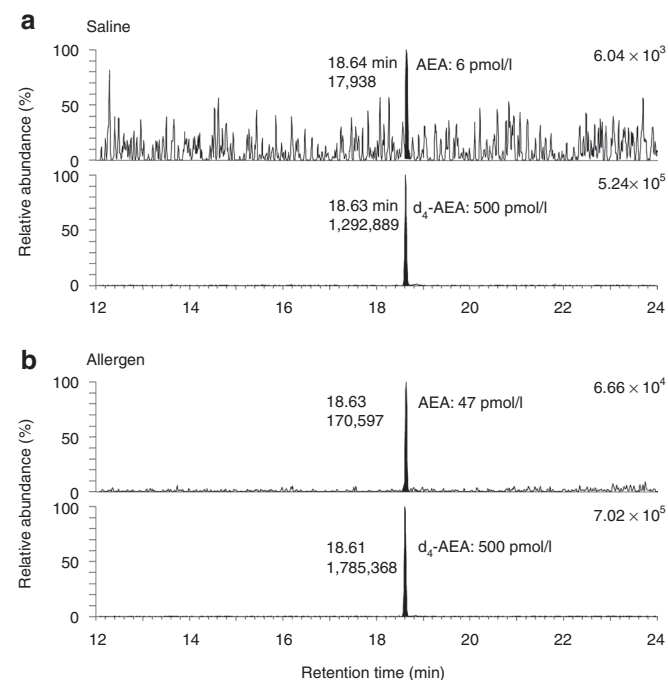


Figure 2 Gas chromatography–mass spectrometry/mass spectrometry chromatograms from representative bronchoalveolar lavage fluid samples showing a concentration of unlabeled anandamide (d₀-AEA) slightly above the limit of detection (a) after saline exposure and a sample from the same patient (b) after allergen exposure.

concentrations. **Figure 2** shows representative gas chromatography–mass spectrometry/mass spectrometry chromatograms and individual anandamide concentrations in BAL samples. Bronchoalveolar lavage anandamide concentrations in saline-stimulated segments were 4.2 ± 0.9 pmol/l. In patients with anandamide concentration levels below the detection limit of

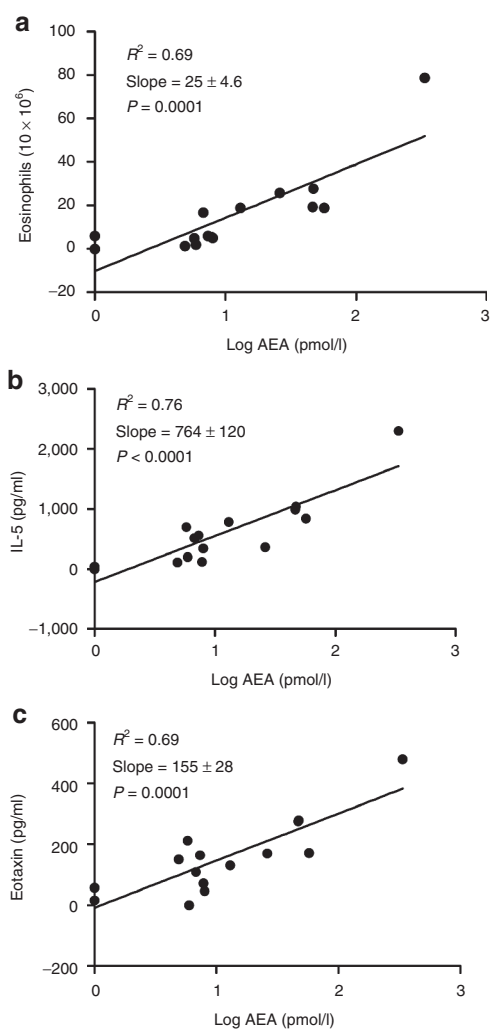


Figure 3 Correlations between anandamide (AEA) concentrations and (a) eosinophilic granulocyte counts, (b) interleukin (IL)-5 concentrations, and (c) eotaxin concentrations in bronchoalveolar lavage samples after allergen provocation. The data were analyzed using unweighted linear regression.

1 pmol/l, the concentration was set to 1 pmol/l for statistical analysis. Allergen provocation increased anandamide concentrations to 38.3 ± 21.8 pmol/l ($P = 0.01$) (**Figure 1d**).

We further assessed whether anandamide concentrations were related to the magnitude of experimental airway inflammation (**Figure 3**). In BAL fluid, anandamide concentrations significantly correlated with eosinophilic granulocyte counts and IL-5 and eotaxin concentrations. Finally, we determined whether eosinophilic granulocytes could be a source as well as a target for anandamide. Eosinophilic anandamide concentrations in intracellular blood were ~ 56 pmol $\pm$ 1.3 pmol per 1×10^9 eosinophilic cells. Analysis of gene expression in blood eosinophils relative to 18S rRNA revealed the presence of CB2 receptors but not CB1 receptors. Furthermore, mRNA from the anandamide synthesizing and degrading enzymes *N*-acyl phosphatidylethanolamine phospholipase D and FAAH were present in eosinophilic granulocytes. We have therefore shown that all major anandamide signaling components are present in eosinophilic granulocytes.

DISCUSSION

The main finding of our study is that experimental allergic airway inflammation in patients with asthma is associated with an activation of the ECS, marked by an increase in bronchoalveolar anandamide concentrations. Moreover, anandamide concentrations were positively correlated with allergic airway inflammation markers. Finally, we showed that human eosinophilic granulocytes express important genes of the ECS and produce anandamide. Our clinical data confirm preclinical findings relating to the involvement of the ECS in allergic asthma.^{8,9}

A particular strength of our study is the standardized experimental model used to elicit local allergic airway inflammation by applying saline and allergen to different lung segments. Through this approach, the pathophysiology and clinical pharmacology of allergic asthma can be studied on an individual basis. We have previously applied the approach to show that endotoxin augments allergic airway inflammation in human subjects¹³ and to test the safety and efficacy of locally acting drugs.¹⁴ Another strength of our study is the rigorously validated gas chromatography–mass spectrometry/mass spectrometry–based assay, which allows for sensitive anandamide measurements in small sample volumes.¹⁵ High sensitivity is required because BAL dilutes pulmonary anandamide concentrations. This methodological aspect also explains the very low anandamide concentrations in the picomolar range in BAL fluid.

We observed a robust increase in bronchoalveolar anandamide concentrations with allergen exposure and strong correlations of anandamide concentrations with eosinophilic granulocyte count as well as with IL-5 and eotaxin concentrations. Eosinophilic granulocytes, IL-5, and eotaxin are important biomarkers and mediators of allergic airway inflammation, and increased numbers/concentrations can be observed in patients with asthma.^{12,16} Therefore, our study suggests that there is an involvement of the ECS in the pathogenesis of human allergic asthma. Whether the increase in anandamide production is a cause or consequence of allergic airway inflammation cannot be discerned. However, the ECS can inhibit as well as promote inflammatory responses in animals.^{17,18} Improvement with anandamide⁹ and the non-specific CB1/CB2-agonist CP55,940⁸ in experimental airway disease suggests that anandamide may indeed regulate allergic airway inflammation by acting on CB receptors. The specific CB2 agonist, WIN 55,212-2, was also shown to suppress antigen-induced airway inflammation in guinea pigs.¹⁹

Anandamide also affects inflammatory processes through mechanisms that are independent of the CB receptor. These are reduction in tumor necrosis factor- α release, NF- κ B inhibition, and attenuation of NO₂⁻ production.^{20–22} We therefore speculate that local release of anandamide could serve as an inhibitory feedback mechanism, attenuating allergic airway inflammation through CB receptor-dependent as well as CB receptor-independent actions. However, anandamide concentrations in the micromolar range could lead to tissue damage, as shown in cardiomyocytes.¹⁸

In addition to providing insight into the components of the pathophysiology of allergic asthma, our study suggests that the ECS could be explored as a potential therapeutic target. Currently,

academic researchers and pharmaceutical companies are pursuing the development of selective CB2 agonists for the treatment of pain and inflammation.^{23–26} Given the abundant CB2 expression in eosinophils, CB2 modulation via selective CB2 agonists could be a novel strategy for the treatment of inflammatory airway diseases, including allergic asthma. Pharmacological FAAH inhibition is a reasonable alternative strategy. However, excessive availability of anandamide through FAAH inhibition might elicit cough mediated by transient receptor potential vanilloid-type channel 1.²⁷ Furthermore, FAAH inhibition may promote CB1-mediated tissue damage.¹⁸ In either case, systemic manipulation of the ECS may elicit psychiatric or metabolic side effects,^{28–30} making a local treatment strategy more appealing.

METHODS

Nonsmoking subjects 22–47 years of age with mild intermittent allergic asthma sensitized to house dust mite or grass pollen (according to the asthma classification defined at the GINA Report 2006, revised³¹) were included in the study. The inclusion criteria were a positive skin prick test for grass mix or *Dermatophagoides pteronyssinus* at or within 12 months prior to the screening visit and a positive airway response to an incremental inhaled allergen challenge. The subjects used β -2-adrenoreceptor agonist inhalers for symptomatic relief as needed. Those with a history of allergen immunotherapy and/or treatment with corticosteroids, sodium cromoglycate, theophylline, or leukotriene modifiers within the 4 weeks before the commencement of the study were excluded. The Hannover Medical School ethics committee approved the study protocol. Written informed consent was obtained from each subject before study entry.

After premedication with 200 μ g of inhaled salbutamol, 0.05–0.1 mg/kg intravenous midazolam, and local anesthesia with topical lidocaine, bronchoscopy (using a BF 160 P bronchoscope; Olympus Optical, Tokyo, Japan) was conducted using a standardized method. During each subject's first bronchoscopy, a baseline bronchoalveolar lavage was obtained by instilling five aliquots of 20 ml sterile normal saline (37°C) into the lung. The lavage fluid was immediately aspirated and collected on ice. Before allergen instillation, 10 ml normal saline (control) was instilled into a right-upper-lobe segment (typically R3) of the lung. Next, the lingula segments (L4, L5) and one segment of the middle lobe (typically R5) were each instilled with 10 ml allergen extract (mixed grass pollen or house dust mite) in sterile saline. The individual allergen dose was calculated on the basis of the skin prick test dilution series performed during screening. The lowest concentration showing a positive result in the skin prick test was chosen, the minimal allergen dose being 62.5 biological units. Twenty-four hours later, during the subject's second bronchoscopy, bronchoalveolar lavage was performed separately in the allergen-challenged and saline-challenged lung segments.

BAL fluid was filtered through a 100- μ m filter and centrifuged. The supernatant was stored at –80°C. The total nucleated cell count was determined using a Neubauer hemocytometer. Differential cell counts were obtained using Diff-Quick staining (Dade Behring, Marburg, Germany), counting 400 cells per cytospin slide. Bronchoalveolar lavage anandamide levels were determined by gas chromatography–mass spectrometry/mass spectrometry, with slight modifications to the method described above for plasma.¹⁵ Unlabeled anandamide (d₀-AEA) and [²H₄]-anandamide (d₄-AEA) were purchased from Cayman Chemical (Ann Arbor, MI). BAL samples (500 μ l) were thawed and spiked with d₄-AEA as an internal standard at a final concentration of 500 pmol/l. Analytes were extracted with ethyl acetate (1,500 μ l). After centrifugation (4,600g, 5 min, 4°C), the organic phase was transferred into a glass vial, evaporated, and derivatized as described above. The residue was dissolved in heptane (50 μ l) containing 2 vol.% pentafluoropropionic anhydride. Analyses were performed on a triple-stage quadrupole mass spectrometer ThermoElectron TSQ 7000 (Finnigan MAT, San Jose, CA) directly interfaced with a Trace 2000 series gas chromatograph. Chromatographic separation took

place on an OPTIMA-5-ht fused silica column (15 m × 0.25 mm i.d., 0.1 µm film thickness; Macherey-Nagel, Düren, Germany). Quantitative measurements were made by selected reaction monitoring of the common product ion with a mass-to-charge ratio (m/z) of 303 generated by collision-induced dissociation of the precursor ions at m/z 540 for d_0 -AEA and m/z 544 for d_4 -AEA. Study samples were analyzed in four runs, together with 14 quality-control samples, i.e., 500 µl aliquots of a solution of unlabeled d_0 -AEA (500 pmol/l) and the internal standard d_4 -AEA (500 pmol/l) in physiological saline. In quality-control samples, the anandamide concentration was 464 ± 52 pmol/l, with a precision (relative standard deviation) of 11% and a mean accuracy of 93%. The limit of quantification was ~1 pmol/l AEA in physiological saline.

Measurements of IL-5 and eotaxin in BAL samples were conducted using a commercial multiplex kit (Meso Scale Discovery, Gaithersburg, MD) in accordance with the manufacturer's instructions. The percentage of eosinophil granulocytes was determined by differential cell counts on cytopspin slides. Peripheral eosinophilic granulocytes were collected and isolated, in a procedure described previously,¹⁴ from venous blood samples drawn from two patients with allergic asthma. These cells were homogenized in phosphate-buffered saline with a Precellys Dual 24 homogenizer (Bertin Technologies, Villeurbanne, France) to extract anandamide and measure its concentration. From other sets of eosinophilic cells, RNA was isolated by column extraction in accordance with the manufacturer's protocol (RNeasy Protect Mini Kit; Qiagen, Hilden, Germany). RNA quality was determined using the RNA 6000 Chip in the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). RNA concentration was measured by UV absorbance detection (Nanodrop; PeqLab Biotechnology, Erlangen, Germany). For cDNA synthesis from 2 µg total RNA, the High Capacity cDNA FT Kit (Applied Biosystems/Ambion, Darmstadt, Germany) was used. The cDNA was then subjected to real-time PCR (7500 Fast Real-Time PCR System, Applied Biosystems) with gene-specific primers (CB1: #Hs00275634_m1; CB2: #Hs00275635_m1; FAAH: #Hs01038660_m1; and N-acyl phosphatidylethanolamine phospholipase D: #Hs00419593_m1).

All data are expressed as mean values ± SEM. Differences in the results of saline challenge and allergen challenge were compared using the Wilcoxon matched-pairs signed-rank test. A P value <0.05 indicated statistical significance. Pearson correlation coefficients were calculated.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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