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Eosinophil activation and novel mediators in the aspirin-induced nasal response in AERD

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ABSTRACT

Background: Eosinophil activation is the key feature of upper and lower airway inflammation in aspirin-exacerbated respiratory disease (AERD).

Objective: To investigate the mechanism of eosinophil activation and identify novel inflammatory mediators using proteomics.

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Methods: Thirty-two asthmatic subjects were enrolled: 18 AERD patients who showed positive responses to the lysine-aspirin nasal provocation test (L-ASA NPT) and 14 aspirin-tolerant asthma (ATA) patients who showed negative responses to the L-ASA NPT (control group). Nasal lavage fluid (NLF) was collected before (baseline), at 10, 30, and 60 min (early response), and at 3 h (late response) after the L-ASA NPT. Eosinophil cationic protein (ECP) and cysteinyl leukotriene (CysLT) levels were measured using an ImmunoCAP system and ELISA, respectively. To identify proteins involved in AERD, comparative proteomics was applied using NLFs collected before and after L-ASA NPTs in AERD patients. The clinical relevance of identified novel proteins was evaluated by ELISA using NLFs from the AERD and ATA groups.

Results: ECP and CysLT levels both increased significantly during the early response in AERD. ECP levels increased until the late response in AERD, while CysLT levels were not significantly increased during the late response. Proteomic analysis showed up-regulation of apolipoprotein A1 (ApoA1), α 2-macroglobulin (α 2M), and ceruloplasmin (CP), with significant increases in NLF of AERD patients, which was significantly higher in AERD patients with chronic rhinosinusitis. Significant correlations were noted between ECP and CysLT, ApoA1, α 2M, and CP levels during the early response in AERD patients.

Conclusion: Eosinophil activation occurred in early and late responses after L-ASA NPT in upper airway mucosa of AERD patients, where ApoA1, α 2M, and CP as well as CysLT may be involved in eosinophilic inflammation.

Key words: Aspirin-exacerbated respiratory disease (AERD), eosinophil, CysLT, proteomics.

INTRODUCTION

Aspirin-exacerbated respiratory disease (AERD) refers to a spectrum of upper and/or lower respiratory reactions, such as rhinitis, conjunctivitis, and bronchospasm that develop following ingestion of aspirin or other non-steroidal anti-inflammatory drugs. AERD is characterised by a clinical tetrad of aspirin hypersensitivity, bronchial asthma, and chronic rhinosinusitis with nasal polyposis [1]. The prevalence of aspirin hypersensitivity in the general population ranges from 0.6 to 2.5%, and is increased to 10–20% in asthmatics [2]. AERD patients present with more severe clinical symptoms, frequent exacerbation of symptoms, and irreversible airflow obstruction, compared with patients with aspirin-tolerant asthma (ATA) [3-5].

Although the pathogenesis of AERD is not fully understood, the cyclooxygenase theory is widely accepted. Aspirin inhibits the COX pathway and diverts arachidonic acid metabolites to the lipoxygenase pathway with reduction of local biosynthesis of prostaglandin E2. Overproduction of leukotrienes by this change of arachidonic acid metabolism results in broncho-constriction, mucus hypersecretion, and increased vascular permeability in the airways of AERD patients [1, 6]. To-date, however, the sequence of biochemical events regulating the aspirin-induced reaction is not fully understood. With technical development such as proteomics and genomics, new attempts have been made to understand the pathogenic mechanism and develop new biomarkers using these tools. Particularly, proteomics is a technology that identifies individual proteins from all the proteins expressed in a cell or tissue. Protein profile changes in target organs following aspirin provocation in AERD patients provide new insights into understanding the pathogenesis.

Recently, eosinophil activation has been considered to play a key role in the inflammatory response of upper and lower airways of AERD patients. Indeed, bronchial and nasal polyp biopsy specimens from AERD patients reveal extensively increased numbers of eosinophils compared with biopsy specimens from ATA patients [7-8]. Moreover, eosinophils have been identified as a major cell type of arachidonic acid metabolism in airway inflammation [9]. Thus, understanding the mechanism of eosinophil activation in airway inflammation of AERD may provide new insight into the pathogenesis of this disease.

Cysteinyl leukotrienes (CysLTs), potent biological mediators generated from arachidonic acid, have been suggested to be one of the main factors involved in AERD [10-11]. CysLTs have been reported to demonstrate a chemotactic effect on eosinophils [12-15]. This observation suggests that CysLTs are involved in eosinophil activation of airway inflammation in patients with AERD. Additionally, a previous study demonstrated that serum eotaxin-2 levels were higher in the sera of AERD patients [16-17]. Eotaxin-2, which belongs to the CC-chemokine family, was reported to be a chemokine for eosinophil recruitment to the asthmatic airway [18-19]. Thus, eotaxin-2 may be involved in airway inflammation in patients with AERD. Since rhinosinusitis is a common comorbid condition experienced by AERD patients, we applied the lysine-aspirin nasal provocation test (L-ASA NPT) to collect nasal lavage fluid (NLF).

In the present study, we aimed to measure serial changes in ECP, CysLT, and eotaxin-2 levels in NLF during L-ASA NPT to investigate the mechanism of eosinophil activation in nasal inflammation after

aspirin challenge. Additionally, we adopted a proteomics-based approach to identify novel inflammatory mediators involved in the pathogenesis of AERD.

METHODS

Study subjects

Thirty-two asthmatic patients who met the criteria for asthma as defined by the Global Initiative for Asthma (GINA) guidelines [20] were enrolled in the present study. The phenotype of AERD and ATA was based on certain histories of ASA/NSAID hypersensitivity and the results of the L-ASA bronchoprovocation test that was performed in all subjects as described previously [16, 21]. All subjects were given the L-ASA NPT and then classified into two groups: 18 AERD patients (AERD group) who showed positive responses to the L-ASA NPT and, as a control group, 14 ATA patients with rhinitis (ATA group) who showed negative responses to L-ASA NPT. The subjects' demographic and clinical data were collected. Baseline characteristics of the study group are presented in Table 1. Their atopic status was investigated by the skin prick test using common inhalant allergens. All subjects were non-smokers. Those subjects with allergic fungal sinusitis, respiratory infection during the previous 4 weeks (purulent sputum or rhinorrhoea, fever) or a history of immunotherapy were excluded from the present study. All subjects were clinically stable. No subject had received a systemic steroid. Topical nasal steroids, antihistamines, and leukotriene receptor antagonists were withdrawn for 1 week before the study. Doses of asthma medications, including inhaled steroids and rescue drugs, were not changed. Patients with nasal polyps of grades 2 and 3 did not participate in the study as they could not undergo reliable rhinometry measurements. Diagnosis of chronic rhinosinusitis was made based on the patient's clinical symptoms, documented medical histories, endoscopic findings, and mucosal changes of maxillary and ethmoid sinuses on radiologic findings [22]. The study was approved by the Institutional Review of Board of Ajou Medical Center, Suwon, Korea, and each subject provided written informed consent.

NPT and nasal lavage

Intranasal challenges were performed with L-ASA solution using a modified protocol that was described previously [23-25]. Shortly after the 30-min adaptation period, the subjects were given a saline challenge to exclude nasal hyper-reactivity. In the absence of a non-specific hyper-response, 80 μ L L-ASA solution containing 12 mg of L-ASA was administered in one nostril by direct instillation with a micropipette. To evaluate nasal response, the nasal symptoms for rhinorrhoea, nasal congestion, sneezing, and itching or

burning of the nose or ear and acoustic rhinometry (ARS 1003; SGS, North Miami Beach, FL, USA) were measured in the first 30 min at 10-min intervals. The clinical score of symptoms was expressed with the following scale: 0, none; 1, mild; 2, moderate; and 3, severe. If the nasal response was negative, the administered dose of L-ASA was increased to 24 mg and the response was observed. If the response was still negative, then 24 mg L-ASA was repeatedly administered (total cumulative dose was 60 mg L-ASA). A positive response was defined based on two following criteria: 1) an increase of more than three points in the total nasal symptom score, a sum of the above individual scores (maximal score = 12 points), and 2) a $\geq 25\%$ decrease in the total nasal volume value, compared with the baseline value as assessed by acoustic rhinometry [24-25]. An early response was defined as that occurring until 60 min and a late response was defined as that occurring at 3 h after NPT [21].

Nasal lavage was performed bilaterally at baseline, and then at 10, 30, and 60 min (early response), and finally at 3 h (late response) after NPT, as described previously [26]. Sterile saline (5 mL) was instilled into each nostril using a syringe. The measurement of recovered fluids showed an approximately 83% recovery with a range of 68–90%. To maximise cell viability, the samples were immediately placed on ice and centrifuged at $300 \times g$ for 10 min. The supernatants were immediately frozen (-80°C) for further analysis.

Two-dimensional electrophoresis (2-DE), image analysis, and mass spectrometric analysis

To identify the proteins involved in the airway inflammation of AERD, NLF samples obtained before and 30 min after the L-ASA NPT in two patients with AERD were used. After each sample had been prepared by trichloroacetic acid/acetone precipitation for desalting and concentration, 2-DE was performed as described previously [27]. Briefly, 1 mg of protein per sample was applied to immobilised pH 3–10 nonlinear gradient strips (Amersham Pharmacia Biotech, Uppsala, Sweden). Isoelectric focusing was performed at 80,000 Vh. The second dimension was analysed on 9–16% gradient polyacrylamide gels until the dye front reached the lower end of the gel. The gel was stained with Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories, Hercules, CA, USA) and scanned using a GS-710 imaging densitometer (Bio-Rad). After converting into electronic files, the images were analysed using Image Master Platinum 5 (GE Healthcare, Piscataway, NJ, USA).

For mass spectrometry (MS), protein spots were excised from the gels, cut into smaller pieces, and digested with trypsin (Promega, Madison, WI, USA) as described previously [28]. Analyses of peptide masses and data processing of spectra were performed using the model 4800 MALDI-TOF/TOF analyser (Applied

Biosystems, Foster City, CA, USA) and Data Explorer version 4.4 software (PerSeptive Biosystems, Framingham, MA, USA), respectively. Peptide matching and protein searches were performed by database searching of the Swiss-Prot and National Center for Biotechnology Information (NCBI) databases with the Matrix Science (<http://www.matrixscience.com>) search engine.

Time-course assay of ECP and inflammatory mediators in NLF during the L-ASA NPT

ECP levels in the NLF samples were measured by immunoassay using the Pharmacia CAP system (Pharmacia-Upjohn, Uppsala, Sweden). CysLT levels were determined in NLF at all time points using a commercially available EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA). The CysLT concentration in NLF was expressed as the total amount of LTC₄, LTD₄, and LT E₄. The lower detection limit of the kit was 13 pg/mL. Eotaxin-2 levels in the each NLF sample were also measured using a commercially available ELISA kit (R&D Systems, Abingdon, UK). The inter- and intra-assay coefficients of variations of this ELISA kit are 8.0% and 5.4%, respectively.

To evaluate the relevance of novel inflammatory proteins identified using proteomics, three proteins apolipoprotein A1 (ApoA1), α 2-macroglobulin (α 2M), and ceruloplasmin (CP) were selected due to their suggested role in the inflammatory process and availability of validation studies. The serial changes in the NLF and serum level of each selected protein were measured using a commercially available ELISA kit [Assaypro (MO, USA), Immundiagnostik AG (Bensheim, Germany), and Assaypro, respectively]. For ApoA1 and CP, NLF samples were diluted 1:6 and 1:10, respectively. The concentrations of all mediators were calculated from the standard curve generated from the standards. The inter- and intra-assay coefficients of variation in ApoA1, α 2M, and CP were 7.3% and 4.8%, 9.23% and 6.20%, and 7.0% and 4.0%, respectively. The detection limits of the assays were 3 ng/mL for ApoA1 and 100 ng/mL for CP.

Statistical analysis

Values are given as the means $\pm$ standard errors. The *t*-test and chi-square test were used for comparisons between the groups. Wilcoxon's test was also used to compare the results in NLF obtained for the time-course provocation test within the group. Pearson's correlation test was used to evaluate the relationships between ECP and other inflammatory mediators. All analyses were performed using the SPSS 13.0 software (SPSS Inc., Chicago, IL). In all analyses, a *P* value less than .05 was considered to be statistically significant.

RESULTS

Changes in the rhinitis symptom score during the L-ASA NPT

Intranasal challenge with L-ASA, but not with saline solution, induced clinical symptoms, including rhinorrhoea, nasal congestion, sneezing, and nasal or eye itching. In the AERD group, the patients began to present nasal symptoms at 10 min and showed the maximal response at 30-60 min after the L-ASA NPT (early response), which was persistent until 3 h after the L-ASA NPT (late response; Fig. 1). Only two patients showed positive responses after a cumulative dose of 60 mg of L-ASA was administered, whereas all others showed positive responses after 24 mg of L-ASA was administered. The mean cumulative dose of L-ASA to induce a positive response was 38.53 ± 7.57 mg. Sixteen of 18 AERD patients showed positive responses within 30 min after the provocation, and one patient showed nasal symptoms at 3 h after the provocation. In contrast, significant changes were not observed during the L-ASA NPT in the ATA group.

Time-course of NLF concentrations of ECP, CysLT, and eotaxin-2 during the L-ASA NPT

The baseline ECP level in NLF was not significantly different between the two groups; however, it increased at 30 min and 1 h after the L-ASA NPT in the AERD group and was persistent for up to 3 h. Significant changes were noted in ECP levels during the early and late responses in the AERD group ($p = 0.011$ and $p = 0.021$, respectively), while no significant change was noted in the ATA group (Fig. 2).

The baseline CysLT level was not different between the two groups; however, it increased significantly at 30 and 60 min after the L-ASA NPT in the AERD group. CysLT levels were significantly increased during the early response ($p = 0.039$), but not during the late response in the AERD group (Fig. 2). No significant change was noted for the ATA group. The baseline eotaxin-2 level tended to be higher in the AERD group compared with the ATA group with no statistical significance. Moreover, no significant change was noted during the L-ASA NPT in both the AERD and ATA groups.

Proteomic analysis of NLF

To investigate novel inflammatory mediators involved in development of AERD, proteomic analysis was conducted using NLF samples obtained before and after the L-ASA NPT in subjects with AERD. The 2-DE results are shown in Fig. 3. Image analysis was performed for 453 grouped protein spots, of which 101 increased and 63 decreased more than two-fold based on a comparison of the mean expression level before and after the L-ASA NPT. Among them, 15 increased and three decreased spots were selected for matrix-assisted laser

desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The results of these analyses are summarised in Table 2. Apolipoprotein A, ApoA1, α 2M, CP, proapolipoprotein, hypothetical protein, human serum albumin complex form, fibrinogen gamma chain, Sp110 nuclear body protein, transthyretin, hCG19123, erythrocyte membrane protein band, and ZNF 684 protein were increased in NLF after the L-ASA NPT. The decreased spots were identified as squamous cell carcinoma antigen, annexin 1, and psoriasin.

Time-course of NLF concentrations of ApoA1, α 2M, and CP during the L-ASA NPT

To validate proteins identified by MALDI-TOF MS, we measured serial changes in the levels of three proteins, ApoA1, α 2M, and CP, in NLF from AERD and ATA groups. The ApoA1, α 2M, and CP levels increased significantly during the early response, not the late response, in the AERD group ($p = 0.011$, 0.018 , and 0.013 , respectively). However, no significant change was observed in the ATA group (Fig. 3).

Correlations between ECP levels and inflammatory mediators

We evaluated the association of ECP with CysLT and three inflammatory mediators validated in NLF. A significant correlation was found between the ECP level and CysLT during the early response in the AERD group ($r = 0.813$, $p < 0.05$), but not during the late response. Furthermore, significant correlations were found between the levels of ECP and the three inflammatory mediators ApoA1, α 2M, and CP during the early response, but not during the late response in the AERD group ($r = 0.821$, 0.761 , and 0.929 , respectively; $p < 0.05$; Table 3), whereas no significant association was found in the ATA group.

Comparison of ApoA1, α 2M, and CP levels according to the presence of chronic rhinosinusitis and/or nasal polyps in AERD patients

When we compared the levels of these three proteins according to the presence of chronic rhinosinusitis and/or nasal polyps in AERD patients, ApoA1 and α 2M levels increased significantly during the early response and decreased at the late response ($p < 0.05$, respectively; Fig. 5). The CP level showed similar patterns, although statistical significance was not noted ($p = 0.062$; Fig. 5). The ECP level increased during early and late response in AERD patients with chronic rhinosinusitis and/or nasal polyps than in those without it ($p = 0.50$ and $p < 0.05$, respectively; Fig. 5).

Measurement of serum ApoA1, α 2M, and CP levels in AERD and ATA patients

We compared serum levels of ApoA1, α 2M, and CP proteins between AERD and ATA groups, and found no significant difference between them (data not shown; $p > 0.05$, respectively).

DISCUSSION

The key pathogenic mechanism of AERD is overproduction of CysLTs with reduction of PGE2 [1, 29-30]. Eosinophil infiltration with mast cells in the upper and lower airways is a key feature of AERD, although the exact mechanisms are not fully understood [30-32]. To the best of our knowledge, this is the first investigation to analyse serial changes in ECP, CysLT, and three potential protein markers in NLF after the L-ASA NPT in AERD patients compared with ATA patients. First, we confirmed nasal symptom changes during the early response that persisted until 3 h. The ECP level in NLF increased during the early response, which persisted until the late response in the AERD group, indicating that eosinophil activation is a consistent finding in nasal inflammation induced by aspirin exposure. Moreover, CysLT production increased significantly during the early response, but not in the late response in the AERD group. Previous studies have shown that baseline CysLT levels in saliva, sputum, blood, and urine were significantly higher in AERD patients than in ATA patients [9, 33]. However, in the present study, no difference was found in the baseline CysLT level in NLF between the two groups, but a significant increase in CysLT was noted during the early response, in close correlation with the ECP level in AERD patients. No significant change was noted in ATA patients. Eotaxin-2, a chemokine for eosinophils in upper airway inflammation in AERD patients did not change in NLFs after nasal aspirin provocation. Moreover, tryptase, a marker of mast cells in NLF, was not detected. These findings confirmed that eosinophils and CysLTs are the key inflammatory cells and mediators responsible for persistent upper airway inflammation found in AERD patients.

Chronic persistent rhinosinusitis is a key feature of AERD, in which intense eosinophilic infiltration was found in nasal mucosa [29-30, 34]. CysLTs may contribute to the accumulation of eosinophils in airway inflammation [13, 35], since they have been reported to have chemotactic activity for eosinophils *in vitro* and *in vivo* [13-15], and to enhance their survival [36]. The source of CysLTs found in NLF in the present study could be mast cells, which can be activated immediately after aspirin exposure [30, 37]. These findings lead us to suggest that CysLTs released from mast cells are associated with eosinophil activation in upper airway mucosa of AERD patients after aspirin exposure. Previous studies have suggested other mediators, including eotaxin-2,

IL-8, IL-5, CCL5, CCL7, and CCL11, to be involved in eosinophil activation in AERD patients [14, 38-40]. Previous studies showed that increased expression of eotaxin-2 was noted in nasal polyp tissue [38], and increased levels of eotaxin-2 with the CRTH2 genetic polymorphism were noted in sera of AERD patients compared to sera of ATA patients [16]. In the present study, when we measured eotaxin-2 and IL-8 levels in NLF during the L-ASA NPT, the baseline level of eotaxin-2 tended to be higher in AERD patients, but no significant change was noted during the L-ASA NPT in both the AERD and ATA groups. Additionally, IL-8 levels in NLF did not show any significant difference between the two groups, and did not change in NLF after L-ASA NPT (data not shown). Thus, eotaxin-2 may involve in basal activation status of eosinophils in upper airway mucosa of AERD patients, whereas CysLTs is the key mediator to activate eosinophils in response to aspirin exposure. Moreover, when we compared CysLT and ECP levels according to the presence of rhinosinusitis, it increased significantly in AERD patients with chronic rhinosinusitis and/or nasal polyps compared to in those without them. These findings suggested that CysLTs is the key mediator of induction of eosinophil activation in chronic persistent rhinosinusitis and nasal polyps in AERD patients.

The present study is the first to demonstrate the presence of three novel inflammatory mediators, ApoA1, α 2M, and CP, in the NLF of AERD patients using a proteomics approach. Although two reports of a proteomic approach using sera and nasal polyp tissue to compare AERD and ATA groups exist [41-42], to the best of our knowledge, this is the first study to show serial changes in novel proteins differentially expressed in NLF before and after the L-ASA NPT that compared AERD and ATA groups. Moreover, we validated these three proteins in the NLF of each patient by comparing the AERD and ATA groups. Although other proteins were identified in this proteomic data, the clinical relevance of ApoA1, α 2M, and CP was evaluated due to their role in inflammatory processes and their availability for further validation studies. First, ApoA1, a major component of high-density lipoprotein in the plasma, plays roles in cholesterol transport and regulation of inflammation [43]. In the present study, the ApoA1 levels increased significantly in NLF during early response in AERD patients, while no changes were found in ATA patients. Moreover, the ApoA1 level was significantly higher in AERD patients with chronic rhinosinusitis and/or nasal polyps than in those without it. These findings suggest that the ApoA1 in NLF may be a biomarker of chronic rhinosinusitis in AERD patients. In addition, a significant correlation was found between ApoA1 and ECP levels, both of which were collected during the early response. When we compared the serum ApoA1 level between AERD and ATA levels, no significant difference was found between the two groups. Recent studies have reported that ApoA1 had anti-inflammatory effects in a

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mouse model of asthma [44-45]. Although we were unable to elucidate the precise role of ApoA1 in AERD, we speculate that ApoA1 released in NLF may play a protective role against the eosinophilic inflammation that occurs in the chronic rhinosinusitis associated with AERD. Second, α 2M was suggested to be a biomarker protein of AERD since its concentration in NLF increased significantly during the early response, which was correlated with the ECP level at this stage. Although α 2M, a major carrier protein, has been used as a marker of plasma leakage, it can bind and regulate activities of various bioactive peptides, including platelet-derived growth factor, transforming growth factor, and ECP [46-48]. Additionally, a previous study reported that α 2M rapidly lost its anti-proteolytic potential in the presence of either stimulated neutrophils or eosinophils [49]. The possibility of a pathogenic role of α 2M in the present study seems to be low. Instead, we speculate that α 2M is secreted into NLF by increased vascular permeability following aspirin exposure and may augment airway inflammation, since it may lose its anti-proteolytic potential due to concurrent eosinophilic inflammation in NLF. Finally, CP, a 132-kDa protein, is a multicopper-binding plasma protein that exhibits the ferroxidase activity necessary for conversion of toxic ferrous iron to the safer ferric iron. As an acute-phase protein, CP is induced following the inflammation process and acts as an anti-inflammatory agent limiting oxidative stress [50]. Serum CP levels have been reported to be elevated in asthmatic patients [51-52]. We found a significant increase in the CP level in the NLF of AERD subjects, which was correlated significantly with the ECP level. Although the role of CP in the inflammatory process of asthma and AERD is incompletely understood, we speculate that CP in the NLF of AERD patients may inhibit eosinophilic inflammation, exerting a protective effect and suppressing airway inflammation.

In the present study, other upregulated and downregulated proteins, such as proapolipoprotein, fibrinogen gamma chain, Sp110 nuclear body protein, annexin1, and psoriasin, were identified in the NLF of AERD subjects after L-ASA NPT. A previous proteomic analysis suggested that these proteins were involved in inflammatory processes [47, 48]. Additional studies are needed to evaluate the clinical relevance of each protein in AERD.

In conclusion, we confirmed eosinophil activation and CysLT production after aspirin exposure in AERD patients that was persistent until the late response. Additionally, ApoA1, α 2M, and CP may represent biomarkers of chronic rhinosinusitis in AERD patients.

Disclosure of a conflict of interest: The authors declare that they have no conflict of interest.

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TABLES

TABLE 1. Clinical characteristics of the asthmatic patients.

	AERD group (n=18)	ATA group (n=14)	<i>P</i> value
Age (years)	35.56 ± 12.40	32.71 ± 7.70	0.433
Gender (male/female)	4 (22.2%)/14	3 (21.4%)/11	0.957
Disease duration (years)	3.23 ± 4.20	5.17 ± 5.84	0.411
Atopy (%)	8 (44.4%)	8 (57.1%)	0.476
Total IgE (kU/L)	193.28 ± 235.64	166.55 ± 175.99	0.748
ECP (µg/L)	24.25 ± 24.14	37.60 ± 16.38	0.395
Sputum eosinophil (%)	14.73 ± 32.87 (n=11)	22.16 ± 34.35 (n=6)	0.667
% Predicted FEV ₁	82.34 ± 15.43	88.71 ± 17.14	0.389
PC ₂₀ methacholine (mg/mL)	7.35 ± 13.73 (n=13)	3.09 ± 5.51 (n=8)	0.418
Chronic rhinosinusitis with/without nasal polyp (P/A)	11 (61.1%)/7	3 (21.4%)/11	0.036

AERD, aspirin-exacerbated respiratory disease; ATA: aspirin tolerant asthma; ECP, eosinophil cationic protein; FEV₁, forced expiratory volume in one second; PC₂₀, concentration of methacholine to produce a 20% decrease in FEV₁; P, presence; A, absence.

TABLE 2. List of proteins identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometric analysis.

No	Protein name	Baseline relative intensity	Expression change ratio	gi No.	Score	Peptide match	Theory- mass (kD)	pI	Cov (%)
1	Hypothetical protein	0.011	7.5	51476390	72	18	69357	5.88	26
2	Human serum albumin complexed form	0.010	3.9	78101694	66	17	66402	5.57	32
3	α2-macroglobulin	0.022	5.9	46812315	101	26	166022	6.06	26
4	Ceruloplasmin	0.159	3.1	157831597	119	23	120009	5.41	30
5	Fibrinogen gamma chain	0.012	5.1	119625326	102	16	47344	5.54	46

6	Sp110 nuclear body protein	0.036	4.1	209572781	67	18	78444	9.1	26
7	Transthyretin	0.050	3.7	55669575	67	7	12836	5.33	63
8	hCG19123	0.017	3.2	119575890	68	17	68453	8.84	37
9	Lipid-free human apolipoprotein A-I	0.025	2.1	90108664	94	12	28061	5.27	52
10	Proapolipoprotein	0.005	7.0	178775	182	21	28944	5.45	62
11	Apolipoprotein A	0.123	5.2	14719785	41	5	9762	6.3	67
12	Erythrocyte membrane protein band	0.023	3.8	42716291	69	14	71911	6.19	30
13	ZNF684 protein	0.030	2.7	84798738	67	10	27938	9.47	54
14	Lipid-free human apolipoprotein A-I	0.022	3.8	90108664	180	21	28061	5.27	61
15	Antigen of the monoclonal antibody Ki-67	0.026	3.7	415819	69	25	358526	9.45	12
16	Squamous cell carcinoma antigen	0.045	-6.2	239552	145	21	44507	6.35	40
17	Annexin I	0.017	-2.5	4502101	99	14	38690	6.57	48
18	Psoriasin	0.060	-2.5	12053626	72	12	10410	6.08	73

Cov, Coverage; gi No., genInfo identified; pI, isoelectric point. Expression change ratio indicates the ratio of mean expression level after L-ASA NPT compared to that before L-ASA NPT.

TABLE 3. Correlations between ECP and other mediators during early and late responses.

	ECP (µg/L)		
	Baseline	Early Response	Late Response
CysLT (pg/mL)	$r = 0.117$	$r = 0.813^*$	$r = 0.035$
Apolipoprotein A1 (ng/mL)	$r = 0.089$	$r = 0.821^*$	$r = 0.508$
$\alpha 2$ -macroglobulin (ng/mL)	$r = 0.404$	$r = 0.761^*$	$r = 0.374$
Ceruloplasmin (ng/mL)	$r = 0.131$	$r = 0.929^\dagger$	$r = 0.528$
Eotaxin-2	$r = 0.624$	$r = 0.046$	$r = 0.571$

The correlations were evaluated at the indicated time-points after nasal provocation. Significant correlations were found between the ECP level and CysLT, apolipoprotein A1, $\alpha 2$ -macroglobulin and ceruloplasmin during the early response in the AERD group, but not during the late response. Statistical significance was evaluated by Pearson's correlation test. $^*p < 0.05$, $^\dagger p < 0.001$.

FIGURE LEGENDS

Fig. 1. (A) Changes in the total nasal symptom score during the lysine-aspirin nasal provocation test (L-ASA NPT). Solid circles, AERD (aspirin-exacerbated respiratory disease) group; Open circles, ATA group. (B) Changes in the nasal symptom score during L-ASA in the AERD group. In the AERD group, the patients began to present nasal symptoms at 10 min after the NPT and the majority of patients showed the maximal response at 30 min after the NPT. * $p < 0.05$, † $p < 0.001$.

Fig. 2. (A) Serial changes in ECP, CysLT, and eotaxin-2 levels in nasal lavage fluid during the L-ASA NPT. Solid circles, AERD group; Open circles, ATA group. (B) ECP, CysLT, and eotaxin-2 levels in NLF during early and late responses after the L-ASA NPT. In the AERD group, the levels of ECP were significantly increased during both early and late response, whereas the CysLT level increased only during the early response. The results were expressed as the means $\pm$ standard error. Statistical significance was evaluated by the Wilcoxon signed-rank test. * $p < 0.05$.

Fig. 3. Two-dimensional electrophoresis (2-DE) separation of nasal lavage fluid collected before (A) and after (B) the L-ASA NPT in an AERD patient. Among the protein spots grouped in the two gels, those that increased or decreased in intensity by more than two-fold were analysed using MALDI-TOF MS. Spots 3, 4 and 9 were identified as $\alpha 2$ -macroglobulin, ceruloplasmin, and apolipoprotein A1, respectively.

Fig. 4. (A) Serial changes in apolipoprotein A1 (ApoA1), $\alpha 2$ -macroglobulin ($\alpha 2$ M), and ceruloplasmin (CP) levels in nasal lavage fluid during the L-ASA NPT. Solid circles, AERD group; Open circles, ATA group. (B) ApoA1, $\alpha 2$ M, and CP levels in NLF during early and late responses after the L-ASA NPT. In the AERD group, the ApoA1, $\alpha 2$ M, and CP levels increased significantly during the early response, but not the late response. The results were expressed as the means $\pm$ standard error. Statistical significances were noted by the Wilcoxon signed-rank test. * $p < 0.05$.

Fig. 5. Serial changes in ECP (A), CysLT (B), apolipoprotein A1 (C), $\alpha 2$ -macroglobulin (D), and ceruloplasmin (E) levels according to the presence of chronic rhinosinusitis in AERD patients. Solid circles, subjects with chronic rhinosinusitis and/or nasal polyp; Open circles, subjects without chronic rhinosinusitis and/or nasal polyp. ApoA1 and $\alpha 2$ M levels showed significant increments during the early response in AERD group with chronic rhinosinusitis, also CP level showed similar patterns. Statistical significance was evaluated by the Wilcoxon signed-rank test. * $p < 0.05$.









