

Release of cyclooxygenase-2 and lipoxin A₄ from blood leukocytes in aspirin-exacerbated respiratory disease

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ABSTRACT

Background: The release of cyclooxygenase-2 (COX-2) and lipoxin A₄ (LXA₄) from blood mononuclear cells in patients with aspirin-exacerbated respiratory disease (AERD) is only partially understood.

Objective: To investigate the presence of COX-2 and LXA₄ in peripheral blood mononuclear cells (PBMC) derived from patients with AERD and with nasal polyps (NP) (designated as the AERD-NP group), patients with NP without AERD (the NP group), and healthy controls without sinus disease (the control group).

Methods: Blood was taken from 14 patients in the AERD-NP group, 6 patients in the NP group, and 8 healthy subjects in the control group. After culturing of human PBMC, the presence of COX-2 protein and LXA₄ (ELISA) was detected in the supernatant, and the results were compared among the groups.

Results: COX-2 and LXA₄ were detectable after culturing of PBMC in all patients in the AERD-NP and NP groups and in the control subjects. COX-2 was highest in the patients in the AERD-NP group, but the difference was not significant compared with patients with non-AERD polyp and with the control subjects. LXA₄ was also highest in the AERD-NP group, but the difference was also not significant compared with the patients who were non-AERD polyp and the control subjects.

Conclusion: Neither the release of COX-2 or LXA₄ was different between the patients with AERD and with NPs, the patients without AERD and with NPs, and the healthy control group. The release of these proteins in AERD needs further investigation.

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Aspirin-exacerbated respiratory disease (AERD) is a clinical syndrome of upper and lower airway inflammation. Patients typically have chronic eosinophilic rhinosinusitis with nasal polyps (NP), asthma, and hypersensitivity to drugs that nonselectively inhibit cyclooxygenase (COX) 1 and COX-2 enzymes, such as acetylsalicylic acid (ASA) and other nonsteroidal anti-inflammatory drugs (NSAID).¹ Patients with AERD and NPs often need sinus surgery. The rate of polyp recurrence is higher than in patients tolerant to aspirin and NSAIDs.² However, the etiology of chronic rhinosinusitis with NPs in patients who are aspirin intolerant still remains unclear.^{3–7}

AERD is caused by an abnormal metabolic shift of arachidonic acid (AA) toward the lipoxygenase-pathway and overproduction of cysteinyl leukotrienes (LT). AA is the primary substrate of the “arachidonic acid” cascade, which results in proinflammatory and anti-

inflammatory mediators. COX, an enzyme also called prostaglandin (PG) H₂ synthase, plays a key role in the conversion of AAs to PGs and thromboxane A₂. Conversion takes place through two distinct reactions: the COX activity, which transforms AAs into PGG₂ and peroxidase activity, which further converts PGG₂ to PGH₂. PGH₂ is then transformed to thromboxane A₂ and PGs by specific synthases.⁸ PGE₂ has been shown to block eosinophil and mast cell degranulation through the prostaglandin E2 and prostaglandin E4 receptors.^{9,10}

Two isoforms of COX are currently known. Whereas COX-1 is a constitutive or “housekeeping” enzyme expressed in most tissues, COX-2 is an “inducible” isoform. The expression of COX-2 can be induced by various substances, such as lipopolysaccharides, cytokines (e.g., interleukin [IL] 1 β , tumor necrosis factor α), phorbol esters (12-O-tetradecanoylphorbol-13-acetate) in fibroblasts, endothelial and epithelial cells, monocytes and/or macrophages, synoviocytes, osteoblasts, and T cells.¹¹ Inhibition of COX activity is associated with shunting of AA from biosynthesis of PGs to LTs, i.e., the “5-lipoxygenase pathway,” as it is known in AERD.¹ Lipoxin A₄ (LXA₄) derives from AA via the “5-lipoxygenase pathway.” AA is oxygenated by 5-lipoxygenase to 15-hydroxyeicosatetraenoic acid. This product is then converted by 5-lipoxygenase and epoxide hydrolase to generate LXA₄, a biologically active lipoxigenase interaction product. LXA₄ stimulates

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neutrophil chemotaxis and inhibits natural killer cell cytotoxicity. It also inhibits leukocyte-dependent inflammation.¹² Leukotriene B₄ and other mediators of the 5-lipoxygenase pathway are proinflammatory mediators that are markedly elevated in chronic neutrophilic inflammation.¹²

A study showed conflicting findings on the up- and downregulation of COX-1 and COX-2 enzymes in polypoid and nonpolypoid nasal mucosa and peripheral blood mononuclear cells (PBMC).³ A study demonstrated that eukaryotic transcription nuclear factor κ B light chain-enhancer of activated B cells is involved in the regulation of COX-2.¹³ However, to our knowledge, the mechanism of release of COX-2 from PBMCs is not yet clearly understood. In AERD, PGE₂ levels are reduced, which reflects diminished expression of COX-2 in NPs.¹⁴ In patients who are aspirin tolerant, a spontaneous upregulation of COX-2 messenger RNA was found.¹⁵ COX-2 messenger RNA levels, PGE₂ concentrations, and COX-2 expression in aspirin-intolerant NP tissue were decreased.^{16–20} No differences in COX-2 staining of columnar epithelium and submucosal glands of NPs between AERD and patients who were aspirin tolerant were found.²¹

COX-2 expression and microsomal PGE₂ synthase 1 may be inhibited by IL-4, which thus induces reduced secretion of PGE₂.²² PGE₂ release from PBMC in patients with AERD was significantly decreased compared with controls.²³ In a recent study,²⁴ various signal pathways on COX-2 regulation in AERD were investigated. No specific alterations of the mitogen-activated protein kinases phosphorylation and nuclear translocation dynamics of transcription factors, such as nuclear factor κ B light chain-enhancer of activated B cells and CCAAT-enhancer binding protein, were found.²⁴ LXA₄ was released to a lower extent from stimulated whole blood cells in both patients with AERD and patients with asthma and who were aspirin tolerant.²⁵ A nonsignificant difference in the reduction of epi-lipoxygenase production by whole blood cell stimulation between patients with AERD and patients with asthma and who were aspirin tolerant was observed.²⁵

With this background, the following questions were addressed in this study: (1) Do human PBMC from patients who were aspirin intolerant release COX-2 enzymes in a different manner than those from patients who were aspirin tolerant or from healthy volunteers; and (2) is the LXA₄ release from human, non-stimulated PBMCs from patients who were aspirin intolerant different from that derived from patients who were aspirin tolerant or from healthy volunteers? Thus, in the present study, we analyzed the release of COX-2 and LXA₄ from PBMCs that were not stimulated with lipopolysaccharides by comparing samples from subjects with AERD and NPs with samples from subjects without AERD but with NPs and samples

Table 1 Characteristics for the patients and control subjects

	AERD-NP Group	NP Group	Control Group
No. subjects	14	6	8
No. women/men	6/8	1/5	4/4
Age range, y	28–53	25–60	21–49
No. patients with known ASA hypersensitivity	14	0	0
No. patients with positive oral aspirin provocation result	14	—	—
No. patients with positive skin-prick test result	5	3	2

AERD = Aspirin-exacerbated respiratory disease; NP = nasal polyp; ASA = acetylsalicylic acid.

from healthy individuals. In the case of significant findings, a feasible future use of these values for screening or selection of patients with AERD should be considered.

METHODS

Participants

Patients with AERD who underwent functional endoscopic sinus surgery for nasal polyposis were included in the study (AERD-NP group). All the patients had experienced asthma attacks after ingestion of ASA. AERD was diagnosed by oral aspirin provocation test 6 to 8 weeks after sinus surgery, as reported elsewhere by the same working group.²⁶ Only test results of patients with postoperatively performed positive oral aspirin provocation were included in the final analysis of this study. Patients who were aspirin tolerant and who underwent sinus surgery because of obstructing NPs were considered control patients with NP (NP group).

None of the patients of the NP group had bronchial asthma or evidence of AERD. They had used ASA or other NSAIDs for treatment of headaches or other conditions several times within the past 2 years without any problems. Patients with known immunodeficiency disorders or who had used topical or systemic corticosteroids were excluded. Patients who underwent septoplasty or septorhinoplasty because of anatomic variations and who did not have any sinus problems were considered control subjects (control group) (Table 1). They also had used ASA or other NSAIDs for treatment of headaches or other problems several times within the past 2 years without any problems. Blood of the patients and control subjects was taken before nasal surgery. At the time of surgery, none of the patients

and control subjects was taking corticosteroids or NSAIDs. All the subjects agreed to participate in the study, which was approved by the institutional review board of the University of Ulm.

Culture of Human PBMCs

Fifty milliliters of venous blood was collected into a syringe that contained 8 mL of acid citrate dextran; the sample was immediately placed on ice, and all subsequent steps were performed at 4°C to minimize activation of the cells. The white blood cells were separated by density gradient centrifugation by using Ficoll Paque (Amersham Biosciences Europe GmbH, Freiburg, Germany).²⁷ The resulting mononuclear cell layers were removed and washed with 2–4 volumes of cold Hank's Balanced Salt Solution without calcium or magnesium. The cells were resuspended in 1 mL of cold Hank's Balanced Salt Solution, an aliquot was removed; the numbers of cells were counted, and purity was assessed on a hemocytometer. In all subsequent steps of preparation, the purity was >95%. The cells were then seeded in a six-well culture plate. After 3 hours, the medium was changed to a serum-free medium (culturing medium without fetal bovine serum). The cells were then centrifuged for 3 minutes at 1000 × *g* and at room temperature. COX-2 and LXA₄ concentrations in the supernatants were then analyzed by using conventional enzyme-linked immunosorbent assay.^{28,29}

Enzyme-Linked Immunosorbent Assay

COX-2 protein expression and LXA₄ release were evaluated by competitive enzyme-linked immunosorbent assay (human COX-II [Assay Designs, Inc., Ann Arbor, MI], LXA₄ [Oxford Biomedical Research, Inc., Oxford, MI]).

COX-2. The minimal detectable concentration of COX-2 was calculated to be 0.008 ng/mL. Cross-reactivity to human COX-1 was <0.1%.

LXA₄. The minimal detectable concentration of LXA₄ was calculated to be <0.02 ng/mL. Cross-reactivity to human LXB₄ was 1.00%, whereas that to human LTB₄, LTC₄, LTD₄, and LTE₄ was <0.01%.

Data Analysis

The Kruskal-Wallis test (nonparametric analysis of variance) with the Dunn *post hoc* multiple comparisons test was used, with differences considered as significant for *p* < 0.05. Values of *p* < 0.1 were considered a tendency to a difference. Data are given as median values, together with box-whisker plots. Statistical analysis was performed by using the sta-

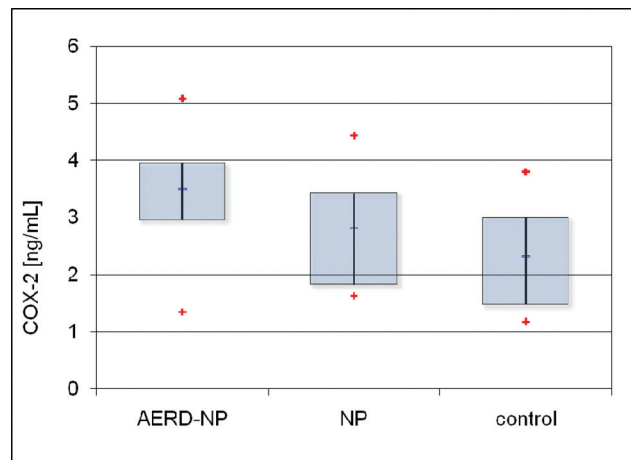


Figure 1. cyclooxygenase-2 (COX-2) content (in ng/mL) after separation of blood mononuclear cells in patients with aspirin-exacerbated respiratory disease (AERD) and nasal polyp (NP) (AERD-NP group), patients with NP without AERD (NP group), and healthy control subjects (control group). The highest concentration was found in patients with AERD. No significant differences were shown (box-whisker plot: -, median; box, upper and lower quartile; +, upper and lower extreme values).

tistical software GraphPad (GraphPad Software, Inc., La Jolla, CA).

RESULTS

COX-2

First, we detected COX-2 concentrations in supernatants of mononuclear cells and compared values from healthy subjects with values from patients with NP, independently of AERD. No differences between healthy subjects and patients with NP were found (*p* = 0.12; data not shown). Values from patients with AERD were compared with the values from the subjects without AERD (*i.e.*, the patients who were aspirin tolerant and with NPs plus the healthy control subjects). Only a slight trend toward increased COX-2 levels in patients with AERD was found (*p* = 0.05). The highest concentration of COX-2 was found in PBMC supernatants of patients with AERD. It did not differ significantly from the concentration of COX-2 in supernatants of patients with NP and without AERD (*p* = 0.34). The concentration of COX-2 in supernatants from the healthy subjects was insignificantly lower than from the NP group (*p* = 0.75). COX-2 in blood from healthy subjects also tended to be lower than from patients with AERD (*p* = 0.12). Overall, there was no suppression of COX-2 enzyme in patients in the AERD-NP group compared with patients with NP and without AERD or the control subjects (Fig. 1).

LXA₄

Analogous to the analysis of COX-2 levels, at first, the LXA₄ concentrations were compared between the

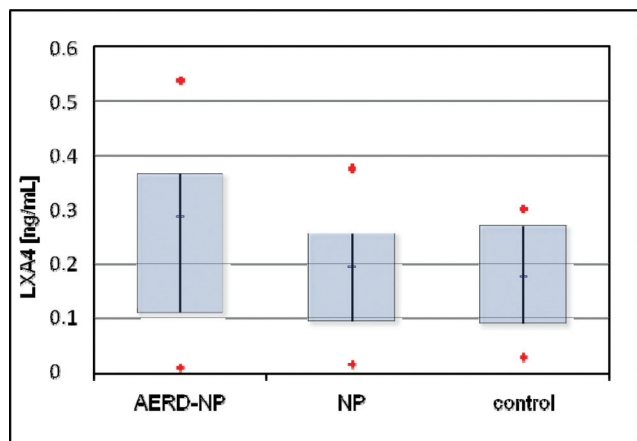


Figure 2. Lipoxin A₄ (LXA₄) content (in ng/mL) after separation of blood mononuclear cells in patients with aspirin-exacerbated respiratory disease (AERD) and nasal polyp (NP) (AERD-NP group), patients with NP and without AERD (NP group), and healthy control subjects (control group). No significant differences were shown (box-whisker plot: -, median; box, upper and lower quartile; +, upper and lower extreme values).

healthy control subjects and patients with NP (*i.e.*, the patients with non-AERD polyp plus the patients with AERD and NPs). No differences between LXA₄ in blood from healthy subjects and from patients with NP were observed ($p = 0.77$). LXA₄ values from the patients with AERD and the subjects without AERD (*i.e.*, the patients who were aspirin tolerant and with NPs plus the healthy control subjects) were compared. No differences between these groups were found ($p = 0.24$). The concentration of LXA₄ in blood from patients with AERD and NP was slightly higher than in supernatants from patients without AERD and with NP ($p = 0.91$) or healthy volunteers ($p = 0.77$). There was no difference in concentrations of LXA₄ between patients in the non-AERD NP group and control subjects ($p = 0.91$). In summary, only a slight trend toward increased LXA₄ concentrations from mononuclear cells from patients with AERD could be found (Fig. 2).

DISCUSSION

In the present study, the release of COX-2, an inducible key enzyme of the PG pathway, and LXA₄, a molecule of the 5-lipoxygenase pathway with hypothesized anti-inflammatory properties, was investigated in patients with AERD and in those without AERD. Release of LXA₄ from AERD PBMCs was insignificantly elevated when compared with patients with NP. The secretion of LXA₄ from AERD PBMC was higher than from the healthy control subjects. However, the difference was not significant. The concentration of COX-2 was highest in blood from patients with AERD. Compared with blood of patients without AERD, the difference was likewise not significant.

COX-2

The present study revealed the highest concentration of COX-2 in PBMC supernatants of patients with AERD. However, the difference to COX-2 in blood of patients with NP and in healthy controls was not statistically significant. One explanation could be that the sample size of our AERD group was too small and that a type 2 error may exist (not finding a difference between blood from the AERD-NP group and blood of patients without that disease). Our observation of elevated COX-2 in blood of patients with AERD-NP is in accordance with the results of Sousa *et al.*³⁰ and Morgan *et al.*³¹ In the study by Sousa *et al.*,³⁰ the number and percentage of mast cells that expressed COX-2 in bronchial mucosa was significantly elevated in subjects with AERD. Morgan *et al.*³¹ showed a COX-2 overexpression in monocytes of patients who were aspirin intolerant after stimulation with aspirin compared with healthy subjects.

In contrast to their findings, downregulation of COX-2 in polyp tissue as well as in bronchial muscular cells from patients with AERD was previously shown.^{32,33} Pujols *et al.*,¹⁶ who also studied the expression of COX-2 in NPs of patients with AERD, found a significant downregulation of COX-2 protein levels compared with non-AERD polyp tissue or nasal mucosa. With the observations in studies with polyps of patients with AERD, a downregulation of COX-2 in blood of the patients with AERD compared with patients with NP but not AERD was expected. We, therefore, believed that other factors than those that triggered regulation of COX-2 expression in respiratory mucosa and polyp tissue were responsible for COX-2-release in blood monocytes.

Upregulation of COX-2 is commonly seen in inflammatory conditions, such as lower respiratory tract infections. Proinflammatory cytokines and bacterial products might be responsible for induction of COX-2.^{12,22} Several studies concluded that downregulation of COX-2 might be involved in the etiology of nasal polyposis.^{24–26} However, other studies revealed conflicting findings with upregulation of COX-2 in NPs of patients without aspirin intolerance.^{27–29} Our finding of slightly upregulated COX-2 in PBMC supernatants of patients with NP (with AERD or without AERD) might be explained by the chronic inflammatory condition of chronic rhinosinusitis with NPs, which contributes to NP development. Steinke *et al.*²² demonstrated that IL-4 significantly inhibits COX-2 and microsomal PGE₂ synthase messenger RNA and protein expression in patients with AERD. It can be hypothesized that IL-4 was responsible for blockage of COX-2 from PBMCs of patients with AERD measured in our study. This, however, was not investigated in the present study.

LXA₄

Our study showed that LXA₄ in PBMC supernatants of patients with AERD were only slightly higher than in patients without AERD. Lipoxins are biologically active eicosanoids that are produced by lipoxygenases. Airway epithelial cells are biologic targets for LXA₄. Little is known about the specific role of LXA₄ in airway epithelial function and pathogenesis of chronic rhinosinusitis with or without NPs.^{15,16} The data of our study supported the hypothesis that LXA₄ may play a role in the resolution of airway inflammation and upper respiratory mucosal damage in AERD. Increased release of LXA₄ from PBMCs of patients with NP may lead to increased upregulation of monocyte chemotaxis and monocyte ingestion of apoptotic neutrophils, reduction of neutrophil-mediated tissue damage, and reduction of neutrophil-mediated inflammation.¹⁴

The expression of interferon γ (IFN γ) on eosinophils in AERD is significantly higher than in tissues from patients without AERD. Thus, LTC₄ synthase expression was significantly increased after IFN γ stimulation, whereas no further increase in LTC₄ synthase was observed in the additional presence of IL-4.³⁴ LXA₄ inhibits the LTC₄ response and may act as an anti-inflammatory in AERD.^{35,36} If IFN γ may also be responsible for reduced expression and secretion of LXA₄ from PBMCs in patients with AERD has to be studied in the future.

Diagnostic Value of COX-2 and LXA₄

In the present study, insignificantly elevated concentrations of LXA₄ and COX-2 in blood of patients in the AERD-NP group were found. Because the production of endogenous LXA₄ is increased when COX-2 is covalently inhibited, *e.g.*, by acetylation from aspirin or other salicylic acids, which thus makes COX-2 behave as a 15(R)-lipoxygenase,¹⁶ elevation of LXA₄ would have been expected in blood of the patients in the AERD-NP group, even when ASA or another NSAID was not administered to the patients before the blood draw. Analysis of the present data, however, did not give evidence that release of COX-2 and LXA₄ is relevantly different in patients with AERD and healthy patients or patients with NPs without AERD.

The factors that trigger regulation and release of both enzymes from human PBMCs are not understood yet. There might be mediators, induced independently of the PG pathway and enzymic metabolism of AA toward prostanoids, which might be responsible for synthesis and release of LXA₄ enzymes from human PBMCs in patients with AERD. The same might be true for the release of COX-2, which was also slightly elevated in supernatants derived from patients in the AERD-NP group. Because lipoxins are synthesized at inflammation sites, direct stimulation and induction of

LXA₄ *via* bacterial products or proinflammatory cytokines might be responsible for secretion from PBMCs.¹⁴ The increased production of LXA₄, however, may alter the prostanoid pathway and the induction of COX-2. However, a more detailed interaction can only be hypothesized and is not supported by this study.

CONCLUSION

This study could not demonstrate that both COX-2 and LXA₄ levels measured after release from human PBMCs may be suitable for monitoring normal and altered eicosanoid patterns from unaffected persons and from patients with eicosanoid-related diseases, *e.g.*, AERD. To our surprise, there was no clear differentiation of the groups investigated, even though there was some evidence that patients with AERD tended to be characterized by an altered COX-2 and LXA₄ pattern as hypothesized in the beginning and derived from some data in the literature on tissue pattern of these enzymes. Further investigation is necessary to understand the role of LXA₄ and COX-2 as part of the complex eicosanoid-protein interaction network that comprises lipid-derived mediators, second messengers, chemokines, receptors, and enzymes.

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