

Reduced expression of COXs and production of prostaglandin E₂ in patients with nasal polyps with or without aspirin-intolerant asthma

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Background: Researchers have debated whether regulation of the COX enzymes (COX-1 and COX-2), which mediate production of prostaglandins (PGs), affects the pathogenesis of nasal polyps (NPs) and aspirin-intolerant asthma (AIA).

Objective: We investigated the roles of PGE₂, COX-1 and COX-2, and PGE₂ receptors in the development of NPs and AIA by measuring their expression in fibroblasts derived from nasal mucosa (NM) and NPs.

Methods: Fibroblasts were isolated from the NM of subjects without asthma who had septal deviation, turbinate hypertrophy, or both (control subjects, n = 7); NPs of aspirin-tolerant nonasthmatic patients (n = 7); and NPs of patients with asthma who were intolerant of aspirin (n = 7). Polyp samples were collected during endoscopic surgery. Cultures were stimulated with IL-1 β (10 ng/mL) for 72 hours. We used ELISA, immunoblotting, and immunofluorescence analyses to measure secretion of PGE₂, expression of COX-1 and COX-2, and expression of the PGE₂ receptors EP1 to EP4.

Results: Compared with NM from control subjects, PGE₂ concentrations were significantly lower in IL-1 β -stimulated fibroblasts from patients with NPs who were tolerant to aspirin and even lower in polyps from patients with AIA. Similarly, IL-1 β exposure induced the expression of COX-1 and COX-2 in fibroblasts from NM of control subjects, had only moderate effects on fibroblasts from NPs of aspirin-tolerant nonasthmatic patients, and almost no effect on fibroblasts from NPs of patients with AIA. IL-1 β also induced expression of EP2 in fibroblasts from control NM but not in fibroblasts from NPs of aspirin-tolerant nonasthmatic patients or those with AIA.

Conclusion: Alterations in the COX pathway (ie, reduced production of PGE₂ and lack of upregulation of COX-1, COX-2, and EP2 under conditions of inflammation) are associated with NPs in patients with or without AIA. (J Allergy Clin Immunol 2011;■■■■:■■■■-■■■■.)

Key words: Aspirin intolerance, asthma, chronic rhinosinusitis, cyclooxygenase, fibroblast, nasal mucosa, nasal polyp, prostaglandin E₂, α -smooth muscle actin

In mammalian cells activation of phospholipase A₂ results in release of arachidonic acid (AA) from membrane phospholipids. AA is in turn transformed by COX and lipoxygenase pathways into eicosanoids. There are at least 2 isoforms of COX enzymes. COX-1 is generally constitutively expressed and considered to be a housekeeping enzyme,¹ whereas COX-2 is induced under chronic inflammatory conditions, such as arthritis,² periodontitis,³ cystic fibrosis,⁴ and chronic obstructive pulmonary disease.^{5,6} COX-2 activation leads to synthesis of several prostaglandins (PGs). Of these, PGE₂ seems to have an important role in inflammatory processes. COX-1 and COX-2 are the targets of classical nonsteroidal anti-inflammatory drugs (NSAIDs), and there are selective inhibitors of COX-2.¹

Bronchial asthma is a chronic inflammatory disorder that, unlike other inflammatory diseases, does not respond to the anti-inflammatory effects of COX inhibitors, such as NSAIDs. In fact, ingestion of NSAIDs even induces bronchoconstriction in some patients,⁷ which is called aspirin-intolerant asthma (AIA). The pathogenic mechanism of AIA is believed to involve altered metabolism of AA, although the putative biochemical defects have not been completely elucidated.⁷ Interestingly, selective inhibitors of COX-2 appear to be well tolerated by patients with AIA.^{8,9} These observations indicate that prostanoids are regulated differently in patients with AIA than in those with other inflammatory diseases.^{7,9} Moreover, these abnormalities in AA metabolism might not occur in only the lower airways because these patients typically have chronic rhinosinusitis with nasal polyps (NPs).^{7,9}

Many studies have reported increased production of cysteinyl leukotrienes in bronchial mucosa and NPs of patients with AIA.^{7,9} Increased release of cysteinyl leukotrienes correlates with upregulated expression of enzymes in the lipoxygenase pathway, particularly of leukotriene C₄ synthase.⁷ Altered expression of COX has also been described in the airways of patients with AIA. However, and in contrast to the reported findings in the lipoxygenase pathway, altered regulation of COX enzymes has

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Abbreviations used

AA:	Arachidonic acid
AI:	Aspirin intolerant
AIA:	Aspirin-intolerant asthma
AT:	Aspirin tolerant
ATA:	Aspirin-tolerant asthma
EP:	Prostaglandin E receptor
NM:	Nasal mucosa
NP:	Nasal polyp
NSAID:	Nonsteroidal anti-inflammatory drug
PG:	Prostaglandin
SFM:	Serum-free media

been reported in some but not all studies; their role in the pathogenesis of AIA is a matter of debate.^{7,9} Several studies that assessed PGE₂ production in patients with AIA produced different results, ranging from reduced production¹⁰⁻¹⁵ to normal production¹⁶⁻¹⁸ or overproduction.¹⁹ Because PGE₂ production requires expression of COX-2 and the PGE₂ synthases, the changes in levels of PGE₂ detected in patients with AIA might result from altered expression of these enzymes. Similar to the disparate results reported in levels of PGE₂ in patients with AIA, COX-2 expression has been reported to be downregulated in the upper airways of these patients²⁰⁻²² and upregulated in airway mast cells²³; other studies did not detect differences in COX-2 expression among patients with AIA, patients with aspirin-tolerant asthma (ATA), patients with chronic rhinosinusitis, or control subjects.^{11,24-26} Furthermore, decreased levels of PGE₂ synthase 1 were detected in nasal tissues of patients with chronic rhinosinusitis.²⁷

Although COX-1 is not considered to be involved in inflammatory responses,¹ some studies found altered expression in airways of patients with aspirin sensitivity,^{11,21,28} and other studies did not report differences.²⁰ PGE₂ signals through 4 G protein-coupled receptors (prostaglandin E receptors 1-4 [EP1-EP4]).²⁹ Expression of EP2 has been reported to be reduced in nasal mucosal inflammatory cells of patients with AIA.³⁰

There are no clear explanations for the reported discrepancies in regulation of COX enzymes in patients with asthma and NPs; they might be caused, at least in part, by differences in study methods.²¹ Regulation of multiple factors in the COX pathway (ie, PGs, enzymes, and PG receptors) has not been assessed in patients with NPs and AIA, and therefore the relationships among different components of the metabolic pathways have not been determined for this disorder. We investigated the levels of the COX enzymes, PGE₂, and EP1 through EP4 in nasal fibroblasts from patients with polyps and from patients with NPs and AIA. Previous studies have shown that alterations in the production of PGE₂ in NPs can occur in the absence of associated asthma.^{21,22} However, isolated nasal polyposis in patients with aspirin intolerance is rarely seen because it is almost always associated with asthma.⁷

METHODS

Nasal mucosa (NM) specimens were obtained from 7 subjects without asthma who had septal deviations, turbinate hypertrophy, or both and underwent corrective surgery (control-NM). All the control subjects had taken normal doses of aspirin or NSAIDs without adverse reactions (asthma, rhinitis, urticaria, angioedema, or anaphylaxis). NP specimens were collected from 7 patients with asthma, chronic rhinosinusitis, and aspirin intolerance

TABLE I. Epidemiologic characteristics of control subjects and patients with NPs

Characteristics	Control-NM fibroblasts	NP-AT fibroblasts	NP-AI fibroblasts
Fibroblast cultures (no.)	7	7	7
Age (y)	33.9 ± 2.9	44.1 ± 16.6	48.1 ± 2.7
Female sex, no. (%)	1 (14.3)	2 (28.6)	4 (57.1)
Asthma (%)	0	0	100
Aspirin intolerance (%)	0	0	100
Atopy, no. (%)	3 (42.8)	2 (28.6)	0 (0)
Blood eosinophilia (%)	2.0 ± 1.2	4.7 ± 3.6	10.0 ± 1.8
Intranasal corticosteroid, no. (%)	0 (0)	6 (85.7)	7 (100)

(NP-AI) who were treated with endoscopic surgery. NP samples were also collected, by using the same procedure, from 7 patients without asthma who tolerated aspirin (NP-AT). The diagnosis of aspirin intolerance was confirmed by means of lysine-aspirin nasal challenge, as previously described.³¹ None of the subjects received oral or intranasal corticosteroids for at least 1 week before the operation or had upper airway infections in the 2 weeks before the operation. All patients provided informed consent to participate in the study, which was approved by the scientific and ethics committee of our institution. Demographic and clinical characteristics of subjects are shown in Table I.

For more information on tissue handling and cell culture; experimental protocols; PGE₂ and COX-2 ELISA, immunoblotting, and immunofluorescence; and statistical analysis, see the **Methods** section in this article's Online Repository at www.jacionline.org.

RESULTS

Culture characterization

Cultures of control-NM, NP-AT, and NP-AI fibroblasts incubated with serum-free media (SFM) for 24 hours did not contain cytokeratin-positive cells, whereas all cells were positive for vimentin (Fig 1). No significant differences were observed in the percentages of myofibroblasts in these cultures (control-NM, 1.5% [25th-75th interquartile, 1.3% to 3.0%]; NP, 4.7% [25th-75th interquartile, 2.4% to 8.7%]; and NP-AIA, 4.5% [25th-75th interquartile, 2.2% to 5.7%]; n = 5).

PGE₂ production

There were no significant differences in the production of PGE₂ at baseline in cultured fibroblasts isolated from control-NM or NP samples. Quiescent cells were incubated with SFM in the presence or absence of 10 ng/mL IL-1β for 24 hours, and PGE₂ secretion was analyzed in culture supernatants collected after 4 and 24 hours. Compared with cells in only SFM, control-NM cells incubated with IL-1β had an increased ratio of PGE₂/total protein after 4 and 24 hours. In contrast, the ratio of PGE₂/total protein did not increase in NP-AT or NP-AI cells after 4 hours incubation with IL-1β, and there was a slight increase after 24 hours. The lowest ratio of PGE₂/total protein was observed in cells from NP-AI tissues; values were significantly different from those of control-NM cells at 4 and 24 hours and from those of NP-AT cells at 4 hours (Fig 2).

COX expression

PGE₂ secretion requires COX-1 and COX-2 activity, and therefore we measured the expression of both enzymes in quiescent

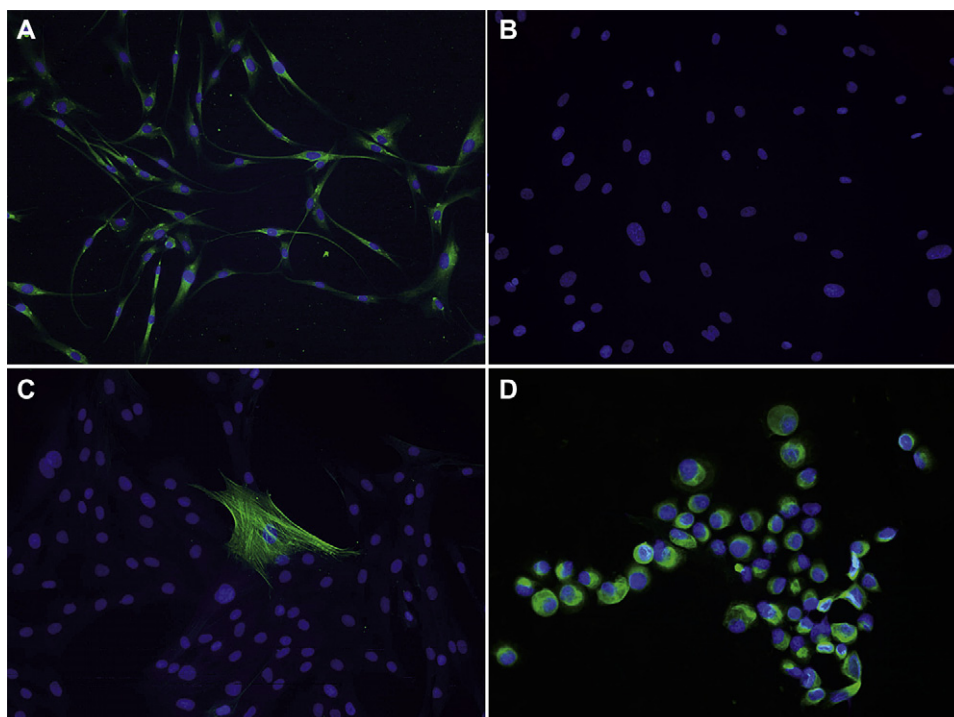


FIG 1. Characterization of cultured cells. Representative images from immunofluorescence analysis of quiescent control-NM fibroblasts. **A**, Presence of vimentin fibers (green) confirms the fibroblast phenotype of cells in culture. **B**, Absence of staining for cytokeratins demonstrates the lack of epithelial cells. **C**, Detection of α -smooth muscle actin fibers confirms the presence of myofibroblasts. **D**, Positive control for cytokeratin in NM epithelial cells (original magnification $\times 200$).

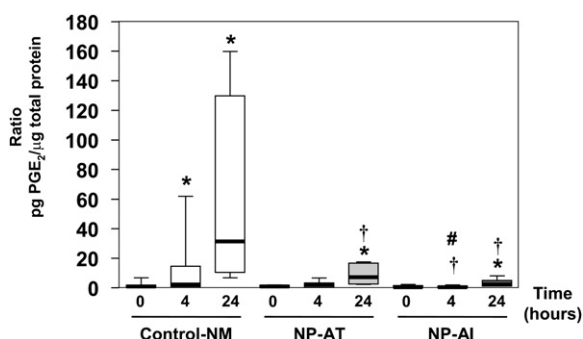


FIG 2. Time course of IL-1 β on secretion of PGE₂ protein. Fibroblasts were incubated with 10 ng/mL IL-1 β . The concentration of PGE₂ ($n = 7$) was measured by means of ELISA. * $P < .05$ compared with control media. † $P < .05$ compared with control-NM fibroblasts incubated with IL-1 β . # $P < .05$ compared with NP-AT fibroblasts incubated with IL-1 β .

fibroblasts incubated in SFM in the presence or absence of 10 ng/mL IL-1 β . Previous studies used various methods to measure COX-2 expression in NP samples and had varying results, and therefore we used several different techniques to quantify expression of COX-2: ELISA, immunoblotting, and immunofluorescence analyses. Measured by means of ELISA, exposure of control-NM fibroblasts to IL-1 β increased expression of COX-2 after 4 and 24 hours. In contrast, the cytokine had a slight nonsignificant effect on the induction of COX-2 in NP-AT and NP-AI fibroblasts. Compared with control-NM samples, the mean increase in the expression of COX-2 protein was significantly lower in NP-AT fibroblasts after 24 hours and in NP-AI fibroblasts after 4 and

24 hours. The lowest expression level of COX-2 was observed in fibroblasts from patients with NP-AI, which was significantly lower than that from NP-AT tissue at 24 hours (Fig 3, A).

In immunoblot analyses, compared with fibroblasts cultured in SFM, IL-1 β increased the ratio of COX-2/ β -actin in only control-NM fibroblasts. COX-2 expression was significantly higher in IL-1 β -stimulated control-NM fibroblasts than in NP-AT or NP-AI fibroblasts at 24 hours (Fig 3, B). Immunofluorescence analysis demonstrated a significant increase in the number of COX-2-positive fibroblasts from all tissues after incubation with IL-1 β compared with cells incubated in SFM. However, the percentage of COX-2 control-NM fibroblasts was significantly greater than that of NP-AT or NP-AI fibroblasts after 24 hours incubation with IL-1 β (Fig 3, C and D).

Although COX-1 is considered a noninducible enzyme, its expression can increase moderately in proinflammatory conditions. In contrast with COX-2, COX-1 protein is very stable and remains in cultured cells for several hours after its synthesis. We measured the effect of 10 ng/mL IL-1 β on expression of COX-1 protein at 24, 48, and 72 hours. Compared with control-NM cells cultured in SFM, control-NM cells incubated with IL-1 β expressed significantly higher levels of COX-1 protein after 72 hours. However, fibroblasts from NP-AT or NP-AI tissues did not alter expression of COX-1 during incubation with IL-1 β (Fig 4).

EP expression

There were no differences in baseline expression levels of EP1 through EP4 among fibroblasts from control-NM, NP-AT, or

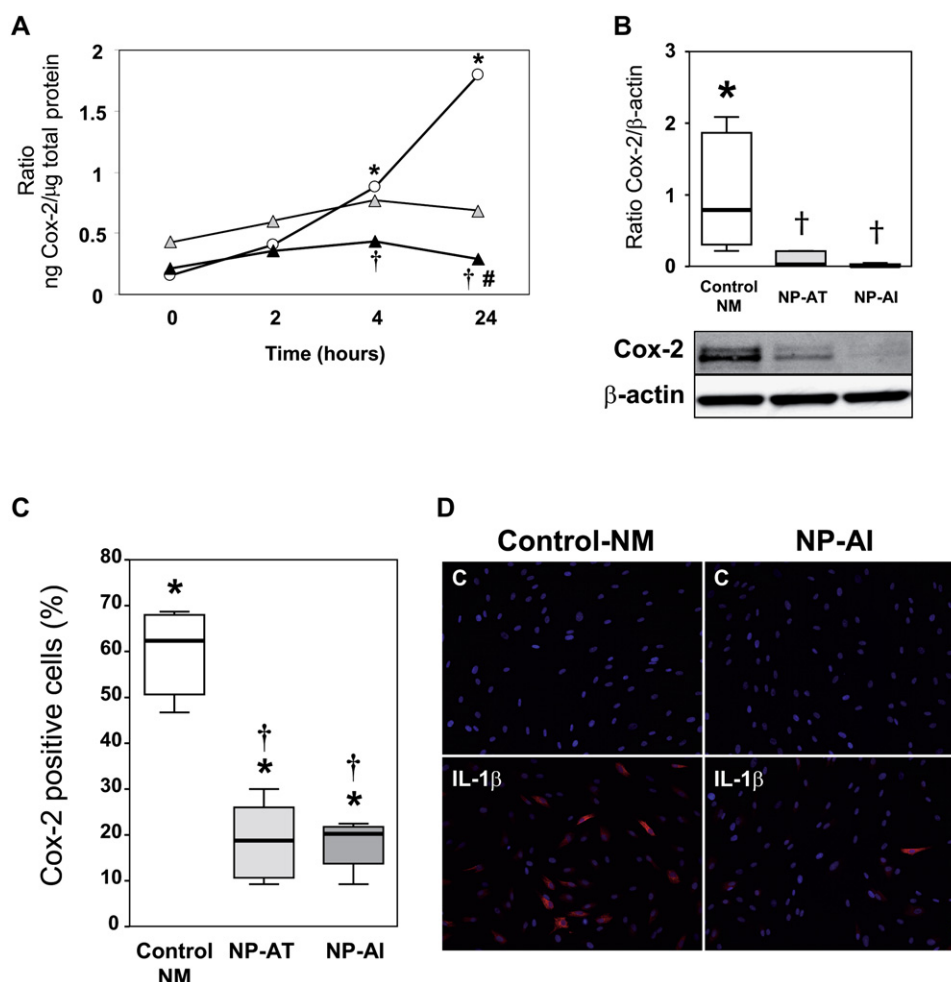


FIG 3. Effect of IL-1 β on expression of COX-2 protein. Fibroblasts were incubated with 10 ng/mL IL-1 β (n = 7). **A**, ELISA analysis of COX-2 protein expression in control-NM (white circles), NP-AT (gray triangles), and NP-AI (black triangles) fibroblasts. **B-D**, Immunoblot (**B**) and immunofluorescence (**C** and **D**) analyses of COX-2 protein (red) after fibroblasts were incubated for 24 hours with IL-1 β . * P < .05 compared with control media. † P < .05 compared with control-NM fibroblasts incubated with IL-1 β . # P < .05 compared with NP-AT fibroblasts incubated with IL-1 β .

NP-AI tissues cultured in SFM (Table II). However, the ratio of EP2/ β -actin increased during incubation of control-NM fibroblasts with 10 ng/mL IL-1 β for 24, 48, and 72 hours. However, this ratio did not increase in fibroblasts from NP-AT or NP-AI tissues compared with that seen in cells cultured in SFM (Fig 5). Expression of EP1, EP3, and EP4 protein did not change in any fibroblast culture during incubation with IL-1 β (data not shown).

DISCUSSION

We investigated secretion of PGE₂ and expression of COX enzymes and EP1 through EP4 in cultured fibroblasts isolated from control NM and NP samples from patients without asthma who are tolerant to aspirin and patients with asthma who are intolerant to aspirin.

We observed that IL-1 β stimulates production of PGE₂ in fibroblasts from control-NM tissues but has a lower stimulatory effect on fibroblasts from NP-AT tissues and hardly any effect in fibroblasts from NP-AI tissues. Expression of COX-2 protein increased after incubation of fibroblasts from control-NM tissues

with IL-1 β but only slightly in fibroblasts from NP-AT tissues and hardly at all in fibroblasts from NP-AI tissues. IL-1 β stimulation increased COX-1 protein expression in fibroblasts from control-NM tissue and to a smaller extent in fibroblasts from NP-AT tissue but did not alter COX-1 expression in fibroblasts from NP-AI tissues. IL-1 β -upregulated EP2 in fibroblasts from control-NM tissues did not induce significant changes in levels of EP2 in fibroblasts from NPs. These results indicate that the COX pathway of AA metabolism is altered in fibroblasts from NPs of patients without asthma and those of patients with AIA.

Altered production of PGE₂ is a characteristic of nasal polypoidosis and AIA; *in vitro* studies have reported reduced production of PGE₂ in epithelial cells from NPs of patients with AIA,¹² bronchial fibroblasts from patients with AIA,¹¹ and peripheral blood leukocytes from patients with AIA,¹³ as well as in NP samples from patients tolerant to aspirin or from patients with AIA.^{14,22} *In vivo* studies have, however, reported varying results, with higher,⁸ similar,^{16,17} or lower,¹⁰ levels of PGE₂ in nasal or bronchoalveolar fluids from patients with AIA compared with patients with ATA or healthy control subjects. These discrepancies

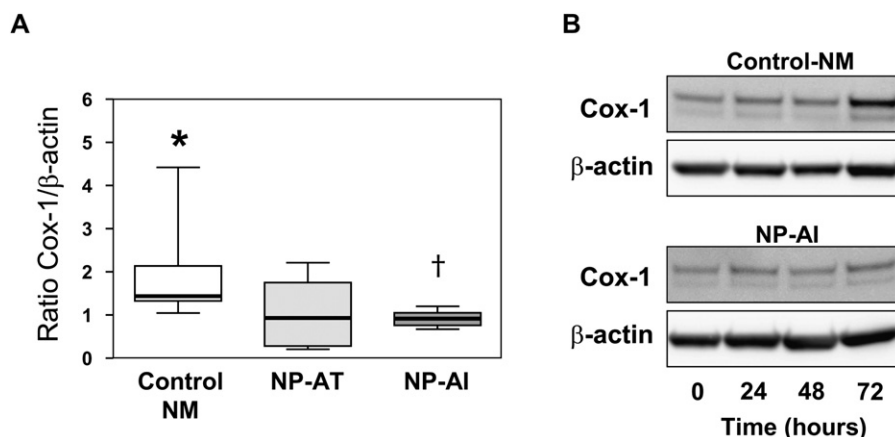


FIG 4. Effect of IL-1 β on COX-1 protein expression. Fibroblasts were incubated with 10 ng/mL IL-1 β for 72 hours (n = 7). **A**, COX-1 protein expression analyzed by means of immunoblotting. **B**, Representative immunoblot. * P < .05 compared with control media (COX-1/β-actin ratio, 1). † P < .05 compared with control-NM fibroblasts incubated with IL-1 β .

TABLE II. Baseline levels of EP1 to EP4 in cultured fibroblasts

PG receptor subtypes	Control-NM fibroblasts (n = 7)	NP-AT fibroblasts (n = 7)	NP-AI fibroblasts (n = 7)
EP1	0.86 (0.66-1.22)	1.44 (0.91-1.91)	1.22 (0.86-1.82)
EP2	0.83 (0.55-1.02)	1.84 (0.62-2.19)	1.11 (0.29-1.39)
EP3	0.88 (0.63-0.96)	1.16 (0.73-1.44)	1.10 (0.82-1.50)
EP4	1.01 (0.91-1.45)	0.95 (0.80-1.54)	1.20 (1.00-1.24)

Data are represented as medians (25th-75th interquartiles) of the EP receptor/β-actin ratio. The Mann-Whitney U test was used for between-group comparisons. No significant differences were found.

probably result from limitations in bronchoalveolar and nasal lavage methodologies and the varying effects of dilution of lavage fluids. The presence of airway obstruction and inflammation in patients compared with control subjects could also affect collection of fluid samples. Studies that quantified levels of PGE₂ in urine had discrepant results: 1 study reported significantly lower concentrations of PGE₂ in urine from patients with AIA compared with those with ATA,¹⁵ whereas another study reported no differences.¹⁸ Studies that measured prostanoid production by using concentrated, exhaled air methodologies reported that patients with ATA and AIA had levels of PGE₂ that were similar to healthy control subjects.³²

Findings of similar levels of PGE₂ among patients with AIA or ATA and healthy subjects have been used to argue against dysregulated PGE₂ synthesis in asthma.^{32,33} However, production of PGE₂ should increase under conditions of inflammation; the similar production of PGE₂ in patients with asthma and healthy control subjects is an anomaly rather than a normal finding. PGE₂ release is increased, however, in other airway inflammatory diseases, such as cystic fibrosis³⁴ and chronic obstructive pulmonary disease.⁶ The prostanoid pathway is therefore abnormally regulated in patients with NPs, especially among those with AIA.

We found that expression of COX-2 increased significantly in NM fibroblasts stimulated with a proinflammatory cytokine (IL-1 β), but this response was reduced in fibroblasts from NP-AT tissue and almost undetectable in fibroblasts from NP-AI tissue. This observation concurs with the findings of Pérez-Novo et al²² and Pujols et al,²¹ who also observed reduced

levels of COX-2 mRNA in NPs, especially from those of patients with AIA. Other studies, however, did not report abnormal regulation of COX-2 in the airways of patients with ATA or AIA. How can these discrepancies be explained? Pierzchalska et al¹¹ reported very low production of PGE₂ in bronchial fibroblasts from patients with AIA but did not detect differences in COX-2 expression in fibroblasts isolated from the same patients, control subjects, or patients with ATA. However, they measured COX-2 levels at only 1 time point (4 hours). Our study and other studies showed the importance of collecting samples at a number of time points to detect changes in levels of COX-2 in patients with AIA; the fact that Pierzchalska et al¹¹ did not use this approach could contribute to their findings.

Studies that used immunohistochemistry to quantify COX-2 expression have yielded contradictory results. In one study COX-2 was reportedly upregulated in mast cells from airways of patients with AIA,²³ whereas other studies reported no differences in expression of COX-2 in cells from upper^{25,26} or lower airways²⁴ of patients with AIA, patients with ATA, or patients with chronic rhinosinusitis compared with healthy control subjects. The static characteristics of immunohistochemical analyses used in these studies²³⁻²⁶ might account for their discrepancies with findings from studies that measured COX-2 kinetics levels by means of immunoblotting or RT-PCR assays.^{21,35} Discrepancies might also result from the use of polyclonal versus mAbs to quantify levels of COX-2.³⁶ The accuracy of immunohistochemical analyses can also be limited by histological differences among tissues; COX-2 is located in the nuclear membrane, and antibodies used to detect it might penetrate the cells of the NM differently from cells from NPs. To overcome these technical limitations, we studied COX-2 expression in isolated and stimulated fibroblasts using ELISA and immunoblot analyses and used immunostaining to confirm observed alterations in COX-2 expression in NP-AT and NP-AI tissues.

COX-1 is considered a constitutively expressed enzyme that responds to the physiological needs of cells but is not involved in inflammatory responses.¹ Some studies, however, reported small increases in its expression in inflamed tissues.³⁷ COX-1 was reported to be substantially upregulated in NPs from patients with chronic rhinosinusitis associated with cystic fibrosis, indicating

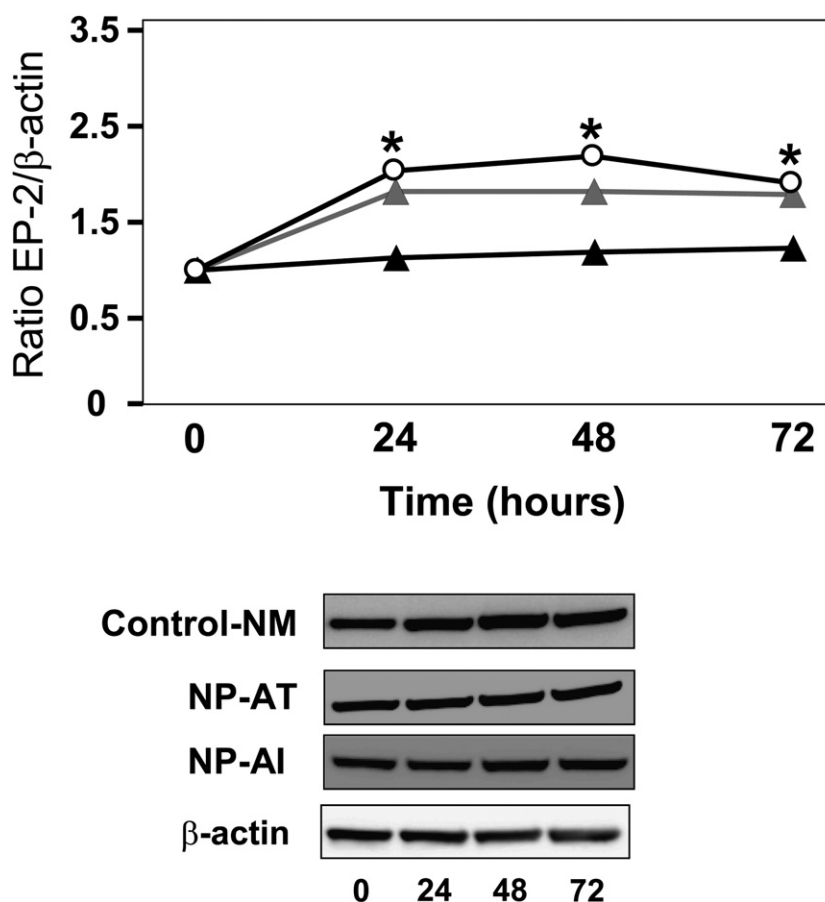


FIG 5. Time course of IL-1 β on EP2 protein expression. Fibroblasts were incubated with 10 ng/mL IL-1 β for 72 hours ($m = 7$). EP2 protein expression in control-NM (white circles), NP-AT (gray triangles), and NP-AI (black triangles) fibroblasts was analyzed by means of immunoblotting. Representative immunoblots are shown. * $P < .05$ compared with control media.

that airway COX-1 is sensitive to inflammatory stimuli,⁴ but studies of the regulation of COX-1 in NPs of patients with and without AI have yielded contradictory results. Some studies reported no differences in COX-1 expression between NM and NPs from patients with ATA or AIA,^{20,26} whereas others found higher levels of COX-1 in NPs from patients with AIA compared with healthy NM.²¹ Pierzchalska et al^{11,28} found significantly lower levels of COX-1 mRNA in bronchial epithelial cells and fibroblast from asthmatic patients compared with healthy subjects, and this difference was greater in patients with AIA. Kinetic studies have shown that COX-1 is upregulated, either spontaneously or after stimulation with cytokines, in samples of NM but not NPs from AT subjects.³⁵

We used stimulated fibroblasts to investigate the regulation of COX-1 in NPs from patients with AIA. Previous studies found a delay between transcription of mRNA and increased levels of COX-1 protein.^{38,39} Interestingly, and in contrast to COX-2 protein, COX-1 protein has a very long half-life.^{38,39} On the basis of these observations, we measured COX-1 protein levels up to 72 hours after fibroblast stimulation with IL-1 β and found increased levels of COX-1 protein in control NM tissue but not in NP-AI tissue. Altered regulation of COX-1, along with the lack of upregulation of COX-2, might contribute to the low levels of PGE₂ detected in NPs from patients with AIA in this and other studies.

PGE₂ has functions in the lung that limit inflammatory responses and control tissue repair.⁴⁰ The ability of PGE₂ to induce or suppress various mechanisms involved in inflammatory responses indicates the complex activities of its receptors.³⁹ EP1 through EP4 each signal through distinct pathways²⁹; some require cell activation,²⁹ and therefore we studied their levels in fibroblasts stimulated with IL-1 β . IL-1 β increased the expression of EP2, but not EP3, EP4, or EP5, in control-NM fibroblasts. In contrast, IL-1 β had no significant effect on EP2 expression in NP-AI fibroblasts, which is in agreement with the results of a previous study reporting a reduced number of eosinophils that express EP2 in NP-AI samples.³⁰ Most of the anti-inflammatory effects of PGE₂ are reportedly mediated by stimulation of EP2.^{29,40} We speculate that reduced release of PGE₂ (which has anti-inflammatory properties in airways) and lower expression of its EP2 receptor increases the inflammatory process in the airways of patients with AIA. Studies are needed to determine whether regulation of EP2 expression contributes to AIA.

In summary, we have demonstrated alterations in the COX pathway of fibroblasts isolated from NPs, especially in those derived from patients with AIA. Observed alterations include very low production of PGE₂, lack of the expected upregulation of COX-2 on stimulation with a proinflammatory cytokine, and differences in the regulation of COX-1 and EP2 in IL-1 β -stimulated

fibroblast from patients with AIA. Alterations in the COX pathway might be involved in the pathogenesis of NPs, asthma, and AI.

Key messages

- **Reduced production of PGE₂, which can have anti-inflammatory and tissue-repairing effects, might contribute to the development of NPs.**
- **Deficient regulation of COX-1 and COX-2 might be involved, at least in part, in the predisposition of some asthmatic patients to exacerbations induced by NSAIDs.**

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METHODS

Tissue handling and cell culture

NM and NP samples were cut into 3×3 -mm fragments and placed in 6-well plates (NUNC, Wiesbaden, Germany) containing Dulbecco modified Eagle medium supplemented with 10% FBS, 100 IU/mL penicillin, 100 μ g/mL streptomycin (Invitrogen, Carlsbad, Calif), and 2 μ g/mL amphotericin B (Sigma, St Louis, Mo). Cultures were kept in a 5% CO₂ humidified incubator at 37°C. When fibroblasts began to proliferate and covered 50% of the well surface, tissue fragments were removed, and the first passage was performed by adding 0.05% trypsin/0.02% EDTA (Invitrogen) for 5 minutes. The reaction was stopped with 10% FBS-supplemented media, and cells were centrifuged (400g for 5 minutes), seeded in 75-cm² flasks (NUNC), and grown to 80% confluence. At passages 3 to 7, fibroblasts were cultured to subconfluence in CultureSlides and 150-cm² flasks (NUNC, Rochester, NY) to perform culture characterization and experimental protocols. Culture characterization was performed by means of immunofluorescence for vimentin (fibroblasts), cytokeratins (epithelial cells), and α -smooth muscle actin (myofibroblasts) in CultureSlides incubated with SFM for 24 hours. The same batch of FBS was used for the whole experimental period, and mycoplasma contamination was tested by means of PCR in all cultures; none were found to be positive.

Experimental protocols

It was reported that COX expression, in response to exogenous stimuli, is more robust in quiescent cells than in proliferating cells,^{E1} and therefore experimental protocols were started when cultures were subconfluent and after incubation with SFM for 24 hours. Then the culture media were changed, and the cells were incubated with SFM in the presence or absence of 10 ng/mL IL-1 β (R&D Systems, Minneapolis, Minn) for different times. Cell lysates were used to analyze COX-1, EP1, EP2, EP3, and EP4 receptor protein expression by means of immunoblot analysis; COX-2 protein expression was measured by using ELISA and immunoblot analyses. In addition, COX-2 protein was studied with immunofluorescence by using CultureSlides.

Culture supernatants and cell lysates

Culture supernatants were collected, centrifuged at 400g for 10 minutes at 4°C, sterilized through 0.22- μ m filters, and stored at -80°C until PGE₂ concentrations were measured. Total protein was obtained by collecting the cells after 2 washes with ice-cold PBS. The cells were centrifuged (1500g for 5 minutes at 4°C) and resuspended in 0.4 mL of ice-cold lysis buffer (Complete protease inhibitor cocktail tablet in 50 mL of 0.05 mol/L Hepes buffer solution, 0.05% vol/vol Triton X-100, and 625 μ mol/L phenylmethylsulfonyl fluoride). Cells were sonicated twice for 15 seconds in a sonifier (Branson, Danbury, Conn) and centrifuged (12,000g for 10 minutes at 4°C).

ELISAs for PGD₂, PGE₂, and COX-2

Concentrations of PGE₂ in supernatants were measured with enzyme immunoassay kits (Cayman Chemical, Ann Arbor, Mich). The assay range was 7.81 to 1000 pg/mL. COX-2 protein concentrations were measured with an enzyme-linked immunosorbent sandwich assay (Zymed Laboratories, San Francisco, Calif). The assay range was 2.15 to 275 ng/mL. Concentrations

of PGE₂ and COX-2 were normalized to the total protein content in the cell lysate from corresponding samples.

Immunoblot analysis

Thirty micrograms of protein extract in loading buffer (NuPAGE LDS sample buffer) was denaturalized in a thermocycler (70°C for 10 minutes), loaded in 7% TRIS-acetate gels, and run at 125 V for 90 minutes in a Novex XCell II Mini-Cell (Invitrogen) to quantify expression of COX-1, COX-2, and PG receptors (EP1-EP4). The proteins were transferred (20 V for 2 hours) to a 0.45- μ m pore-size nitrocellulose membrane, and nonspecific binding sites were blocked with blocking buffer (5% nonfat dry milk and 0.1% Tween 20 in 10 nmol/L PBS) for 1 hour at room temperature in an orbital shaker. Membranes were incubated with the primary antibody against COX-1 (SC-1752, Santa Cruz), COX-2 (SC-1745, Santa Cruz), EP1 (101740, Cayman), EP2 (101750, Cayman), EP3 (101760, Cayman), or EP4 (45863; Abcam, Inc, Cambridge, Mass) in blocking buffer (1:1000); washed 4 times in 0.5% Tween 20 in 10 nmol/L PBS; and incubated with peroxidase-conjugated secondary antibody in blocking buffer (1:3000). After 4 washes, immunoreactive bands were visualized by using a chemiluminiscent method (Supersignal West Pico Chemiluminescent Substrate, Rockford, Ill), and light emissions were detected with the CCD Camera System LAS 3000 (Fujifilm, Tokyo, Japan). Band intensities were quantified with Fujifilm Image Gauge 4.0 Software and normalized to intensities of β -actin and assessed from the same samples.

Immunofluorescence analysis

CultureSlides were washed with PBS, fixed with paraformaldehyde 4% for 15 minutes, and permeabilized with 0.2% Triton for 10 minutes. Nonspecific binding sites were blocked with 1% BSA-PBS for 1 hour. Antibodies against α -smooth muscle actin (M0851; DAKO, Glostrup, Denmark) diluted 1:500, vimentin diluted 1:100 (V5255; Sigma, Saint Louis, Mo), pancytokeratin diluted 1:200 (C2562, recognizing human cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19; Sigma), or COX-2 (SC-1745, Santa Cruz Biotechnology) were added for 1 hour at 37°C. After 3 washes, a fluorescent secondary antibody was added for 1 hour. Nuclei were stained with 4'-6-diiodino-2-phenylindole dihydrochloride (1/10,000 dilution). Finally, slides were mounted with Prolong gold antifade reagent (Invitrogen). Percentages of positive cells were quantified by using fluorescence microscopy.

Statistical analyses

Immunofluorescence data are expressed as medians (25th-75th interquartiles) of positive-cell percentage among total cells. ELISA results are shown as medians (25th-75th interquartiles) of picograms of PG or nanograms of COX-2 per microgram of total proteins. Finally, immunoblot results are depicted as medians (25th-75th percentiles) of band intensities normalized for β -actin. The nonparametric statistical Mann-Whitney *U* test was used for between-group comparisons, and the Wilcoxon test was used for paired comparisons after confirming differences with the Friedman test. Statistical significance was set at a *P* value of less than .05.

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