

Cyclooxygenase 1 and cyclooxygenase 2 expression is abnormally regulated in human nasal polyps

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Background: There is evidence that impairment of prostanoid metabolism might be involved in the pathogenesis of nasal polyps (NPs). Prostanoids are synthesized by 2 cyclooxygenase (Cox) enzymes, one constitutive (Cox-1) and another inducible (Cox-2). **Objective:** The aim of these studies was to investigate Cox-1 and Cox-2 regulation in NPs of aspirin-tolerant human patients compared with that seen in nasal mucosa (NM). **Methods:** Cultured explants from human NPs and healthy mucosa from patients undergoing polypectomy and corrective nasal surgery, respectively, were examined for Cox-1 and Cox-2 expression by means of semiquantitative competitive PCR and Western blotting.

Results: Cox-1 mRNA was spontaneously upregulated in cultured NM but not in NPs. A spontaneous but delayed upregulation of Cox-2 mRNA was found in NPs (24 hours) compared with that seen in NM (6 hours). After cytokine stimulation (IFN- γ , IL-1 β , and TNF- α), the induction of Cox-2 mRNA and protein was also faster in NM (1 hour) than in NPs (4 hours). **Conclusion:** These data showing an abnormal regulation of Cox-1 and Cox-2 in NPs from aspirin-tolerant patients reinforce the concept that prostanoid metabolism might be important in the pathogenesis of inflammatory nasal diseases and suggest a potential role for this alteration in the formation of NPs. (J Allergy Clin Immunol 2002;109:824-30.)

Key words: Cyclooxygenase, nasal polyp, nasal mucosa, IL-1 β , TNF- α , IFN- γ

Prostanoids are produced when arachidonic acid is released from the plasma membrane by means of phospholipases and metabolized by cyclooxygenase (Cox) and specific isomerases. Prostanoid production depends on the activation of the 2 Cox isoenzymes within cells. Cox-1 is present in most cells, and its expression is generally constitutive, whereas Cox-2 expression is low in most cells,

Abbreviations used

Cox: Cyclooxygenase

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

NM: Nasal mucosa

NP: Nasal polyp

SC: Standard control

but it dramatically increases on stimulation. Prostanoid levels are usually low in healthy tissues, but they also markedly increase during inflammatory responses.¹

Because of their presence in inflamed lesions, prostanoids were initially considered as proinflammatory mediators. However, recent studies support the notion that these mediators can both promote and inhibit inflammation. Raud et al² have shown that prostanoid-release inhibition with indomethacin potentiates antigen-induced inflammatory reactions in sensitized hamsters. Similar findings have been observed by Gilroy et al³ in the rat carrageen-induced pleurisy model, in which resolution of the late-phase inflammatory reaction occurs more slowly in animals treated with nonsteroidal anti-inflammatory drugs. An overall anti-inflammatory role for prostanoids has been suggested in models of allergic airway disease.^{4,5} Moreover, PGE₂ has been reported to attenuate some acute inflammatory responses, particularly those initiated by mast cell degranulation.^{2,6}

Nasal polyposis is a multifactorial process that represents a model of chronic airway inflammation of the nasosinusal mucosa,⁷ which affects 2% to 5% of the population in developed countries.⁸ Although many theories have been suggested, the pathogenesis of nasal polyposis is still unknown.⁹ Nasal polyps (NPs) contain activated inflammatory cells, such as neutrophils, lymphocytes, mast cells, and eosinophils, that release a variety of proinflammatory mediators, including cytokines, histamine, PGs, and leukotrienes.

Accumulated evidence shows that PGs are generated in nasal mucosa (NM) and NPs^{10,11} by many cells, including mast cells, eosinophils, and epithelial cells.¹²⁻¹⁵ Elevated levels of cysteinyl leukotrienes and reduced release of PGE₂ have been reported in chronic sinusitis/NPs from aspirin-sensitive patients.^{11,16,17} We have previously reported that downregulation of Cox-2 possibly accounts for the low PGE₂ production in NPs in these asthmatic patients.¹⁸ These observations support the

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hypothesis that aspirin sensitivity and NPs might be the result of an imbalance of arachidonic acid metabolism.

NPs also develop in aspirin-tolerant asthmatic patients and in nonasthmatic patients. In contrast with aspirin-sensitive patients, no differences in the release of PGE₂ and leukotrienes are detected between unstimulated NM and NPs. However, higher and lower increases in the release of leukotrienes and PGE₂, respectively, were found in NPs compared with that seen in NM on arachidonic acid stimulation.¹⁰ This finding suggests that abnormalities in arachidonic acid might also exist in NPs of aspirin-tolerant patients.

We hypothesize that NPs are the consequence of an inflammatory process that is perpetuated by a deficient production of prostanoids involved in the regulation of chronic inflammatory responses. Because prostanoids are released on activation of Cox-1 and Cox-2, our hypothesis also establishes that this deficiency should be due to an abnormal regulation of one or both Cox enzymes. The primary objective of the present studies was to investigate the regulation of the expression of Cox-1 and Cox-2 in a steady state and after cytokine-driven stimulation in aspirin-tolerant patients with NPs and healthy NM.

METHODS

Materials

Penicillin-streptomycin, HEPES buffer, RPMI-1640 medium, Taq polymerase, Moloney murine leukemia virus reverse transcriptase, dithiothreitol, RNase out, random primers, agarose, and PCR primers were from Life Technologies SA (Barcelona, Spain), and 24-well tissue culture clusters were from TPP (Barcelona, Spain). Amphotericin B was from Squibb (Esplugues de Llobregat, Spain). IL-1 β , TNF- α , IFN- γ , and chloroform were from Sigma Chemical Co (Madrid, Spain). Isopropanol was from Panreac (Barcelona, Spain). TRI Reagent was from MRC (Cincinnati, Ohio), and polyclonal antibodies to Cox-1 and Cox-2 and peroxidase-conjugated anti-goat IgG were from Santa Cruz Biotechnology (Santa Cruz, Calif). Ovine Cox-1 and Cox-2 proteins were from Oxford Biomedical Research, Inc (Oxford, Mich).

Patients

NPs were obtained from aspirin-tolerant patients (age, 50 $\pm$ 1.5 years; age range, 14-73 years; n = 40) undergoing nasal endoscopic polypectomy. Patients with aspirin sensitivity, diagnosed by means of either clinical history, aspirin nasal provocation, or both, were excluded. Two (5%) patients had a positive skin prick test response. Sixteen (40%) patients were receiving intranasal corticosteroids (fluticasone or budesonide, 400-800 μ g/d), oral corticosteroids (prednisone, 10-30 mg/d), or both at the time of the operation. NM was obtained from patients undergoing nasal corrective surgery (age, 32.5 $\pm$ 1.9 years; age range, 18-54 years; n = 19). One (5.3%) patient had a positive skin prick test response, and 2 (10.5%) patients were receiving intranasal corticosteroids, oral corticosteroids, or both at the time of the operation. None of the patients had an upper respiratory infection in the 4 weeks before the operation. The authorship institutional review board and ethics committee approved the study.

Tissue handling

A specimen obtained at the time of the operation was immediately snap-frozen in liquid nitrogen and kept at -80°C. Other specimens were obtained by cutting NM and NPs into approximately 100-mg fragments. A mean of 14 specimens (range, 10-22 speci-

mens) were obtained from each NM or NP sample. Specimens were placed in 24-well culture plates with RPMI-1640 medium supplemented with antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 μ g/mL amphotericin B) and glutamine (150 μ g/mL) and incubated in a controlled atmosphere (5% CO₂, 45% O₂, and 50% N₂), at 37°C.

RT-competitive PCR

Cox-1 and Cox-2 mRNA expression were measured with an RT-competitive PCR, as previously described.^{18,19} This method relies on the addition of known amounts of a cDNA competitor molecule (internal standard control [SC]) in the amplification reactions.²⁰ Hybrid primers of Cox-1•glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or Cox-2•GAPDH were used to create PCR products that were cloned and used as internal SCs in the PCR reaction. The RT-competitive PCR was carried out as follows. First, an internal SC, which was basically a GAPDH fragment with the Cox-1 or Cox-2 sequence at both ends, was amplified by means of single PCR with hybrid primers. Second, total RNA was extracted from NM and NP samples and reverse transcribed to cDNA. Finally, after addition of different concentrations of SC to a constant amount of sample cDNA, a competitive PCR was performed with specific primers for Cox-1 or Cox-2, which coamplified Cox-1 or Cox-2 and the internal SC. After amplification, PCR products were resolved by means of gel electrophoresis, and the yields of amplified SC and sample products were quantified.

Construction of internal SC. As previously described,^{18,19} hybrid primers were designed to obtain the internal SC: Cox-1•GAPDH, 5' TGCCCCAGTCCCTGGCCCCGCGCTT•CCACCCATG-GCAAATTCCATGGCA and 3' GTGCATCAACACAGGCGCTTCTTC•TCTAGACGGCAGGTCAGGTCCACC; Cox-2•GAPDH, 5' TTCAAATGAGATTGTGGGAAAATTGCT•CCACCCATG-GCAAATTCCATGGCA and 3' AGATCATCTCTGCTGAG-TATCTT•TCTAGACGGCAGGTCAGGTCCACC. Amplification conditions were as follows: denaturing at 95°C for 1 minute, annealing for 2 minutes at 55°C for Cox-1•GAPDH and 58°C for Cox-2•GAPDH, and extension at 72°C for 1 minute for 35 cycles. These primers amplified a band of 641 bp, which was basically a GAPDH fragment with the Cox-2 or Cox-1 sequences at both ends. Cox-1•GAPDH (SC1) and Cox-2•GAPDH (SC2) fragments were electrophoresed and electroeluted, as previously reported.^{18,19}

Isolation and quantification of target RNA. By using a technique reported elsewhere,^{18,19} total RNAs from NM and NPs were obtained with a TRI-reagent total RNA extraction kit. The RNA integrity was checked with methods previously described. RNA samples were quantified by means of densitometric analysis with respect to 4 known RNA concentrations loaded in parallel, and total RNA was reverse transcribed to cDNA.

Competitive PCR with internal SC. Specific Cox-1 and Cox-2 primers were as follows: hCox-1 (303 bp), 5' TGCCCCAGTCTCTGGCCCCGCGCTT (position 516) and 3' GTGCATCAACACAGGCGCTTCTTC (position 819); hCox-2 (305 bp), 5' TTCAAATGAGATTGTGGGAAAATTGCT (position 573) and 3' AGATCATCTCTGCTGAGTATCTT (position 878). Known amounts of SC1 or SC2 were loaded into each PCR tube reaction with a constant amount of the diluted target cDNA. Three SCs were selected for Cox-1 (10³, 10⁴, and 10⁵ copies) and 4 for Cox-2 (10³, 10⁴, 10⁵, and 10⁶ copies). Competitive PCR reaction was carried out according to a method previously reported.^{18,19}

PCR product analysis. Amplified cDNA (PCR products) was resolved by means of 1% agarose gel electrophoresis and stained with ethidium bromide in Tris borate-EDTA buffer. The amount of Cox-1 or Cox-2 in relative terms to competitors (SC) was quantified and compared by means of densitometric analysis, as previously reported.^{18,19}

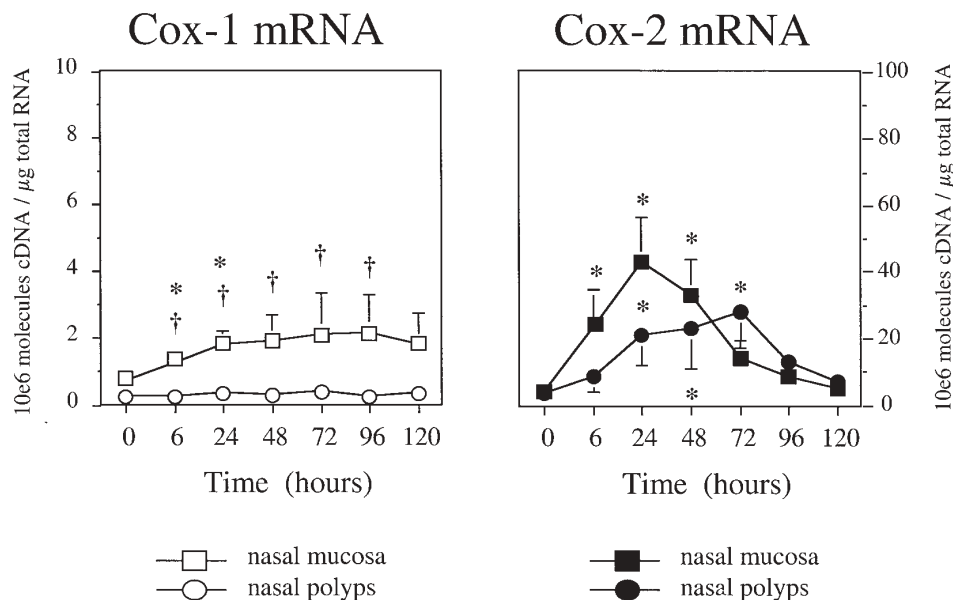


FIG 1. Spontaneous expression of genes encoding for Cox-1 and Cox-2 in NM ($n = 7$) and NPs ($n = 11$). * $P < .05$ compared with the time of the operation (0 hours) and † $P < .05$ compared with NPs (Wilcoxon signed-rank test).

Western blot analysis

Snap-frozen explants taken from NM and NPs under different conditions were frozen, total protein was extracted, and Western blot analyses were performed with specific goat polyclonal IgG anti-Cox-1 or anti-Cox-2 by using methods reported elsewhere.¹⁹ Cox-1 and Cox-2 proteins were used as controls within the assays.

Data analysis

mRNA data are reported as the arithmetic mean $\pm$ SEM of 10^6 molecules of Cox-1 or Cox-2 cDNA per microgram of total RNA. Protein data are reported as the arithmetic mean $\pm$ SEM of the percentage change from Cox standard protein, as measured by means of OD. Nonparametric statistical tests were performed with the Wilcoxon signed-rank test for time-course experiments and Kruskal Wallis analysis with the Mann-Whitney U test for intergroup comparisons in the dose-response experiments. A P value of less than .05 was regarded as statistically significant.

RESULTS

Spontaneous regulation of Cox-1 and Cox-2 mRNA expression

Specimens from NM and NPs were collected and snap-frozen at -80°C at the time of the operation (0 hours) and after being in culture for 6, 24, 48, 72, 96, and 120 hours. Compared with hour 0 (NP, 0.24 ± 0.07 ; NM, 0.67 ± 0.15), spontaneous expression of Cox-1 mRNA in NM ($n = 6$), but not in NPs ($n = 11$), showed a time-dependent upregulation that was significant after 6 hours in culture (Fig 1). Compared with hour 0 (NP, 2.82 ± 0.86 ; NM, 3.26 ± 1.01), NM ($n = 7$) showed a spontaneous Cox-2 mRNA upregulation after 6 hours (22.5 ± 9.5 , $P < .05$), 24 hours (41.9 ± 13 , $P < .05$), and 48 hours (31.7 ± 11.2 , $P < .05$) of culture, whereas in NPs ($n = 11$) Cox-2 was only upregulated after 24 hours (19.9

± 8.6 , $P < .05$), 48 hours (22.2 ± 12 , $P < .05$), and 72 hours (27 ± 8.4 , $P < .05$). A different profile of Cox-1 and Cox-2 mRNA expression was found in NPs with respect to NM. Although it had an early upregulation (6 to 48 hours) in NM, Cox-2 showed a delayed spontaneous upregulation (24 to 72 hours) in NPs.

At the protein level, Cox-1 was constitutively expressed at the time of the operation (0 hours), whereas the level of Cox-2 was almost undetectable. When explants were put in culture, NPs showed a spontaneous upregulation of the Cox-2 protein that was constant and significant after 24 hours ($n = 4$, $P < .05$, Fig 2). Cox-1 protein expression did not change over time in cultured NPs. At baseline levels, no differences were found in Cox-1 and Cox-2 mRNA expression between NPs from patients who were receiving corticosteroid treatment (Cox-1, 0.59 ± 0.15 ; Cox-2, 4.94 ± 0.86) at the time of the operation and those who were not (Cox-1, 0.42 ± 0.13 ; Cox-2, 4.83 ± 1.15).

Effects of cytokines on Cox-1 and Cox-2 expression

A mixture of 3 cytokines (IFN- γ , IL-1 β , and TNF- α) at different concentrations (0.1, 1, and 10 ng/mL) and incubation times (1, 4, 8, and 24 hours) were added to explant cultures to investigate the effect of a proinflammatory stimulus on Cox-1 and Cox-2 expression at both mRNA and protein levels.^{13,19} The cytokine mixture (1 ng/mL) induced the expression of Cox-2 mRNA from 1 to 24 hours in NM and from 4 to 24 hours in NPs (Fig 3). Compared with media-treated explants (NP, 6.2 ± 1.5 ; NM, 7.1 ± 2) and after 1 hour of incubation, cytokines showed a significant effect on Cox-2 mRNA expression in NM (36.1 ± 5.4 , $n = 7$, $P < .01$), but not in NPs (13.6

± 4.7 , $n = 11$, not significant). After 1 hour of incubation, this cytokine-driven effect on Cox-2 expression was also dose dependent in NM (cytokine mixture at 10 ng/mL, 37.8 ± 7.4 ; $n = 6$; $P < .05$), but not in NPs (cytokine mixture at 10 ng/mL, 10.5 ± 2.5 ; $n = 6$; not significant), compared with that seen in media-treated explants (NPs, 6.8 ± 1.7 ; NM, 15.9 ± 1.9 ; Fig 4). However, proinflammatory cytokines were able to cause a dose-related increase in both Cox-2 mRNA and protein expression in NPs after 24 hours of incubation (Fig 5). Proinflammatory cytokines had no effect on Cox-1 mRNA expression from either NM or NPs.

DISCUSSION

Our group previously reported low expression of Cox-2 in NPs of aspirin-sensitive asthmatic patients.¹⁸ We now report that Cox-1 and Cox-2 are abnormally regulated in NPs obtained from aspirin-tolerant patients. The main findings of our study are as follows: (1) Cox-1 mRNA was spontaneously upregulated in cultured NM but not in NPs; (2) a spontaneous but delayed upregulation of Cox-2 mRNA was found in NPs compared with that in NM; and (3) the induction of Cox-2 mRNA and protein was also faster in NM than in NPs after cytokine stimulation.

We have previously reported a similar Cox-1 and Cox-2 expression level in healthy NM.¹⁹ In the present study baseline levels of Cox-2 mRNA in NPs were higher than those of Cox-1 mRNA, probably reflecting the inflammatory nature of NP tissue. In contrast, Cox-1, but not Cox-2, protein levels were detected. The fact that Cox-1 mRNA is a more stable transcript than Cox-2 mRNA might account for this finding.²¹

In keeping with data previously reported,¹⁹ a time-dependent spontaneous upregulation of Cox-1 mRNA was found in NM but not in NPs. Although there is considerable information about how expression of Cox-2 is controlled, little is known on the regulation of the gene encoding Cox-1. It is generally accepted that the expression level of Cox-1 does not greatly vary.²² Nevertheless, Cox-1 expression increases in cell lines that undergo differentiation²² in stimulated lung epithelial²³ and endothelial cells.²⁴

In contrast with Cox-1 mRNA, Cox-2 mRNA was spontaneously upregulated in both NM and NPs. However, the increased expression of Cox-2 was delayed in NPs (24 to 72 hours) with respect to NM (6 to 48 hours). Cox-2 protein from NPs showed a progressive and increasing upregulation after 6 hours in culture, reaching a plateau at 96 hours, and a delayed expression with respect to data previously reported from healthy NM.¹⁹

The cytokine mixture did not cause any change in the expression of Cox-1 mRNA, either in NM or in NPs. This observation supports the concept that Cox-1 has no crucial role in inflammatory responses. However, recent studies have shown that prostanoids formed by means of Cox-1 might be involved in gastric inflammatory reactions.^{25,26} It appears that the degree to which Cox-1 is involved in inflammatory reactions depends on the inflammatory

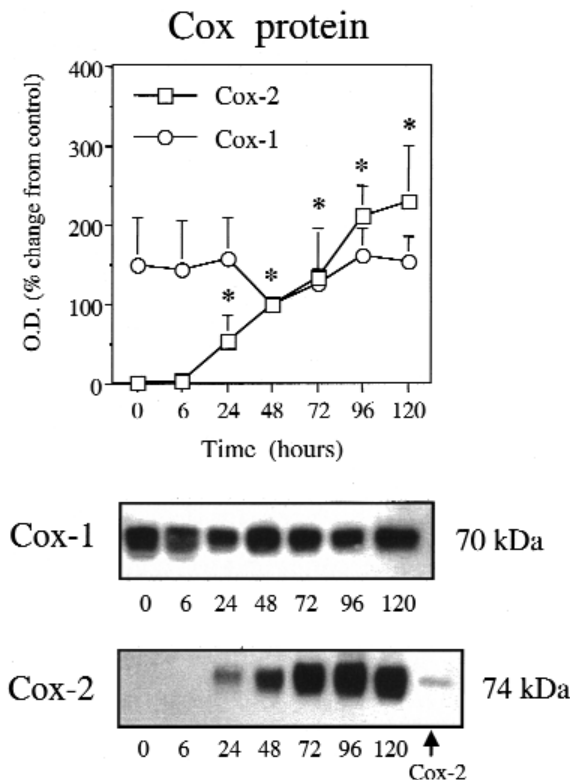


FIG 2. Spontaneous protein expression of Cox-1 and Cox-2 in NPs ($n = 4$). * $P < .05$ compared with the time of the operation (0 hour; Wilcoxon signed-rank test). Insets show representative experiments of Cox-1 (70 kd) and Cox-2 (74 kd) spontaneous protein expression in NPs.

stimulus and the tissue in which the insult occurs.

The cytokines used in our study are potent Cox-2 inducers. Previous studies have reported that TNF- α and IL-1 β cause Cox-2-driven upregulation in isolated rat lungs,²⁷ and IL-1 β induces Cox-2 and PG synthesis in human airway smooth muscle cells.²⁸ A similar cytokine mixture also induced Cox-2 mRNA expression in healthy NM.¹⁹ In our study Cox-2 mRNA dramatically increased when NM and NP explants were exposed to the cytokine mixture. In keeping with the results obtained in the spontaneous induction study, the Cox-2 expression rate in NM was faster (1 hours) than that in NPs (4 hours) after cytokine stimulation.

The mechanisms by which Cox-2 expression is abnormally regulated in NPs have not yet been established. Expression of the gene encoding Cox-2 might be regulated by various cytokines, growth factors, and inflammatory mediators by increasing mRNA transcription and stabilization.^{1,19} In the case of proinflammatory cytokines, a number of signaling pathways are likely to be involved in the regulation of Cox-2 expression, including MAP kinases²⁹ and transcription factors, such as activating protein 1,³⁰ nuclear factor κ B,^{31,32} and nuclear factor IL-6.³³

All our patients with NPs had been treated with glucocorticoids for varying periods of time. Although we did

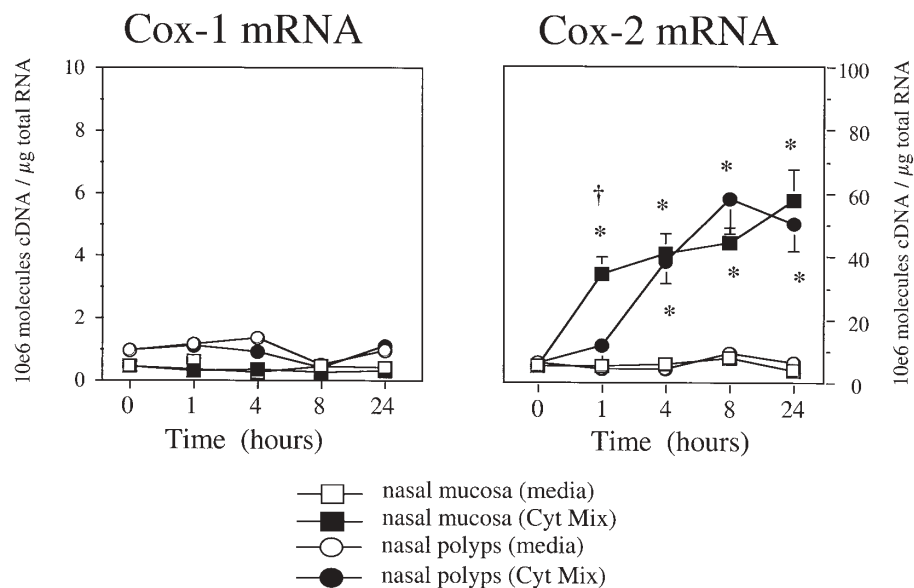


FIG 3. Time-dependent regulation of Cox-1 and Cox-2 mRNA expression by a mixture of proinflammatory cytokines (*Cyt Mix*; IFN- γ , IL-1 β , and TNF- α , 1 ng/mL) in explants from NM (n = 7) and NPs (n = 11). * P < .05 compared with culture media and † P < .05 compared with NPs (Wilcoxon signed-rank test).

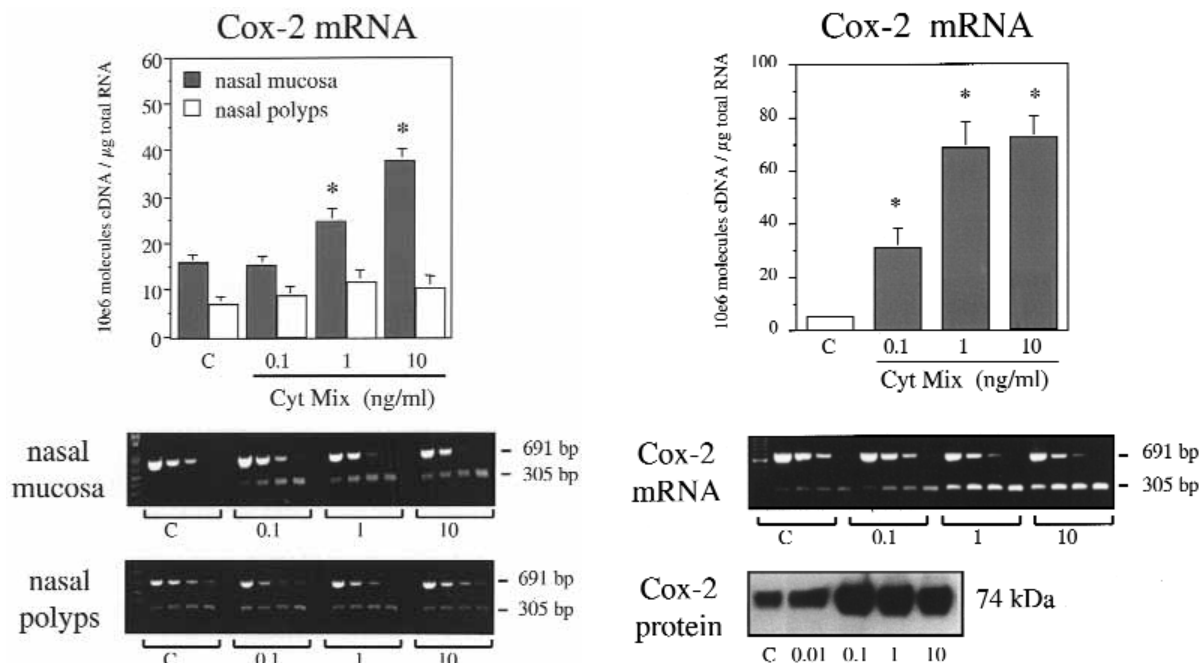


FIG 4. Dose-related effects of proinflammatory cytokine mixture (*Cyt Mix*; IFN- γ , IL-1 β , and TNF- α , 0.1-10 ng/mL) on Cox-2 mRNA expression (1 hour) in explants from NM (n = 6) and NPs (n = 6). * P < .05 compared with culture media (C; Mann-Whitney U test). *Insets* show representative experiments of Cox-2 mRNA expression in both NM and NPs.

FIG 5. Dose-related effects of proinflammatory cytokine mixture (*Cyt Mix*; IFN- γ , IL-1 β , and TNF- α , 0.1-10 ng/mL) on Cox-2 mRNA and protein expression (24 hours) in NPs. * P < .05 compared with culture media (C; Mann-Whitney U test). *Insets* show representative experiments of cytokine-induced Cox-2 mRNA and protein expression in NPs.

not find any differences in either the spontaneous or the cytokine-induced Cox-2 upregulation between patients who were or were not receiving glucocorticoids at the time of the operation, a potential effect of past glucocorticoid therapy on Cox-2 mRNA expression cannot be

completely excluded. Nevertheless, the effects of glucocorticoids on Cox-2 expression are characterized by conflicting observations. In *in vitro* studies, upregulation³⁴ and downregulation¹⁹ of Cox-2 expression has been reported with glucocorticoids. Redington et al³⁵ found

that the expression of Cox-2 was increased in the airway epithelium of non-steroid-treated asthmatic patients compared with that seen in asthmatic patients receiving glucocorticoids.³⁵ In contrast, Dworski et al³⁶ reported that treatment with prednisone increased Cox-2 mRNA and protein in blood monocytes and alveolar macrophages in vivo. The regulation of expression of the gene encoding Cox-2 by glucocorticoids seems to be complex and appears to depend on the cell type and study methods.

Our findings raise important questions about the relationship between abnormalities in the regulation of prostanoid metabolism and the pathogenesis of NPs. Because NPs from patients with aspirin-sensitive asthma express less Cox-2¹⁸ and produce less PGE₂¹⁷ than those from aspirin-tolerant patients, it has been suggested that the insufficient regulation of Cox-2 in aspirin-sensitive asthmatic patients might leave aspirin-sensitive patients without the protective effect of PGE₂, thus accounting for the increased susceptibility to aspirin.¹⁸ We now report that Cox pathways are also abnormally regulated in the NPs of aspirin-tolerant patients. However, the abnormalities are not found in the steady state and are detected only when NPs are removed from the NM or submitted to the stimulatory effect of potent proinflammatory cytokines.

PGE₂ has inhibitory effects on mast cells¹² and eosinophils,^{37,38} and it also prevents allergen-induced asthma,^{38,39} exercise-induced bronchoconstriction,⁴⁰ and aspirin-induced asthma.⁴¹ Intranasal administration of PGE₂ to compensate for the deficient production of endogenous PGE₂ could represent a potential therapeutic alternative in these patients.

In summary, we report that both Cox-1 and Cox-2 expression are differentially regulated in NPs with respect to healthy NM. Cox-1 mRNA was spontaneously upregulated in cultured NM but not in NPs. The rate and intensity of Cox-2 mRNA upregulation, either spontaneous or after cytokine stimulation, was clearly different in NPs compared with that in NM. These data reinforce the concept that prostanoid metabolism might be important in the pathogenesis of inflammatory nasal diseases and suggest an important role for both Cox-1 and Cox-2 pathways in the formation of NPs.

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