

Original article

Nuclear factor- κ B activity is down-regulated in nasal polyps from aspirin-sensitive asthmatics

Background: We examined whether a decreased activity of nuclear factor(NF)- κ B, a transcriptional regulator of cyclooxygenase-2 (COX-2), could account for down-regulation of COX-2 in nasal polyps of aspirin-sensitive asthmatics.

Methods: Nasal polyps were obtained from 17 aspirin-intolerant asthma/rhinitis patients (AIAR; 7 men, mean age 48 ± 12 years) and 23 aspirin-tolerant asthma/rhinitis patients (ATAR; 12 men, mean age 65 ± 11 years). COX-2 mRNA expression was measured using semiquantitative reverse transcriptase competitive polymerase chain reaction (RT-PCR), and the results were expressed as mean $\pm$ standard error of 10^6 molecules of mRNA/ μ g of total RNA. NF- κ B binding was measured with ^{32} P-labeled oligonucleotides and electrophoretic mobility shift assay (EMSA), and the results were expressed as a percentage with respect to the mean EMSA obtained in 19 healthy nasal mucosa.

Results: The mean levels of COX-2 mRNA expression (0.25 ± 0.06) and NF- κ B activity (89 ± 13) in nasal polyps from AIAR were significantly lower than in polyps from ATAR (COX-2 = 1.58 ± 0.50 , and NF- κ B = 143 ± 12 , $P < 0.01$ and $P < 0.05$, respectively). Levels of COX-2 mRNA and NF- κ B activity in polyps from patients on corticosteroid therapy did not differ statistically from those who were not on this therapy before polypectomy.

Conclusion: This study shows that the low expression of COX-2 mRNA in nasal polyps from aspirin-sensitive patients is associated with a down-regulation of NF- κ B activity.

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Key words: aspirin-induced asthma; COX-2; nasal polyps; NF- κ B; rhinosinusitis.

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Accepted for publication 13 August 2002

Chronic rhinosinusitis with nasal polyposis usually develops in almost all aspirin-sensitive asthmatic people (1). There is accumulated evidence to show that metabolism of arachidonic acid appears to be abnormal in nasal polyps of nonallergic drug-hypersensitive patients (2) with aspirin intolerant asthma/rhinitis (AIAR) (3–7). These abnormalities are characterized by low production of prostaglandin E_2 (PGE₂) and increased synthesis of cysteinyl leukotrienes.

Prostaglandins are generated through the cyclooxygenase (COX) pathway. Two forms of COX enzyme are recognized, COX-1 and COX-2. COX-1 represents the constitutive isoform that performs a “housekeeping” function to synthesize prostaglandins, which regulate normal cell activity (8). Inhibition of COX-1 appears to be the key event leading to bronchoconstriction in AIAR (1, 9). COX-2 is the inducible isoform and its expression can increase in response to various stimuli, including cytokines and growth factors (8). Selective COX-2 inhibitors are usually well tolerated by AIAR patients (9, 10).

We have recently reported that COX-2 is markedly down-regulated in polyps from AIAR subjects (11). This abnormality may account for the low production of

PGE₂ in nasal polyps reported by various groups (4–7). The insufficient regulation of COX-2 might also explain the increased sensitivity of some asthma patients to the inhibitory effects of aspirin on COX-1 (11). For some reason, which is as yet unclear, COX-2 appears to protect COX-1 against the effect of aspirin. Since exogenous PGE₂ given by inhalation prevents aspirin-induced asthma (12), it has been suggested that down-regulation of COX-2 may leave aspirin-sensitive patients without the protective effect of endogenous PGE₂, rendering them more susceptible to the inhibitory effect of aspirin (11).

Nuclear factor(NF)- κ B plays a key role in the transcriptional regulation of the expression of inducible genes including COX-2 (13, 14). In the inactive state NF- κ B is kept in the cytoplasm via interaction with various inhibitory proteins known as I κ Bs. Activation of NF- κ B, in response to a variety of proinflammatory signals, leads to the release of the I κ B subunits from the cytoplasmic complex, which transmigrates into the nucleus where it activates its target genes upon binding to various κ B motifs (13, 14). The prototypic NF- κ B heterodimer consists of the p50 and p65 (Rel A) subunits, but other complexes have been described with different members of

the Rel family modulating DNA binding and transactivation (13, 14).

Our interest in the mechanisms involved in ASA-sensitive asthma led us to investigate the role of NF- κ B in the regulation of COX-2 in nasal polyps. In the present study, we hypothesized that a decrease in NF- κ B expression and activation could account for the down-regulation of COX-2 that is observed in nasal polyps from AIAR patients.

Material and methods

Subjects

Nasal polyps were obtained from 17 AIAR patients (7 men; mean age 48 ± 12 year), six of whom were receiving intranasal corticosteroids (fluticasone or budesonide, 400–800 μ g/day) and three of whom were receiving oral prednisone (20–30 mg/day) at the time of surgery; the remaining eight had discontinued intranasal and systemic glucocorticoid therapy at least 2 weeks prior to surgery. The 17 AIAR patients had rhinitis and asthma. The diagnosis of aspirin intolerance was made on the basis of a clear-cut history of asthma attacks precipitated by nonsteroidal antiinflammatory drugs (NSAIDs). In 12 patients, aspirin sensitivity was confirmed by using oral aspirin challenge (two patients) or nasal aspirin challenge (10 patients) according to methods previously reported (15, 16). Five patients with three or more asthma attacks precipitated by various NSAIDs were not challenged.

Nasal polyps were also obtained from 23 aspirin-tolerant asthma/rhinitis (ATAR) patients (12 men, mean age 65 ± 11 year). ATAR patients reported use of various NSAIDs with no adverse effects. Nine were on intranasal corticosteroids (fluticasone or budesonide, 400–800 μ g/day) and three were also on oral corticosteroids (prednisone 15–30 mg/day) when polypectomy was performed. The remaining 11 had discontinued corticosteroid therapy at least 2 weeks before surgery. Among the 23 ATAR patients, 15 had rhinitis alone, and eight had rhinitis and asthma.

None of the patients suffered from upper respiratory infection during the 4 weeks before surgery. All subjects agreed to participate in the study, which was approved by the Ethics Committee of our Institution.

Isolation of total RNA

A specimen obtained at the time of surgery was immediately snap-frozen in liquid nitrogen and kept at -80°C . Total RNA from nasal tissue specimens was isolated using a rapid extraction method (TRI-reagent, MRC, Cincinnati, OH, USA) as previously described (11). The integrity of the purified RNA was determined by visualization of the 28S and 18S ribosomal RNA bands after electrophoresis on a 1% agarose denaturing gel stained with ethidium bromide. Total RNA was quantified by densitometric analysis with respect to four known RNA concentrations that were loaded in parallel.

Reverse transcriptase competitive polymerase chain reaction (RT-PCR)

Total RNA (4 μ g) from nasal mucosa and nasal polyps was reverse-transcribed to cDNA using random hexanucleotide primers and SuperScript II Rnase H⁻ reverse transcriptase. To measure COX-2 mRNA expression we developed a previously reported competitive

PCR technique, in which, after the reverse transcription step, known amounts of an exogenous DNA are added to the amplification mixture (RT-competitive PCR) (11). The exogenous molecule, called competitor or internal standard, was coamplified in competition with the target in the same test tube. Since the initial amount of internal standard was known, this technique permitted the determination of the initial amount of target cDNA.

The specific COX-2 cDNAs were amplified using specific primers whose sequences were as follows: hCOX-2 sense 5' TTC AAA TGA GAT TGT GGG AAA ATT GCT (position 573), and COX-2 antisense 5' AGA TCA TCT CTG CCT GAG TAT CTT (position 878), amplifying a product of 306 bp.

The reverse transcription and PCR reaction conditions have been reported previously (11).

NF- κ B electrophoretic mobility shift assay (EMSA)

EMSA was carried out according to a method previously described (17). Briefly, nuclear proteins were extracted from tissues and 4 μ g of the nuclear protein extract were incubated with ^{32}P -5'-end-labeled double-stranded oligonucleotide (5' AGTTGAGGGGACTTT CCCAGGC-3') containing the consensus NF- κ B site. The NF- κ B complexes were separated on a polyacrylamide gel and transferred on to cellulose paper, dried, and exposed overnight to a film using an intensifying screen. Autoradiographic bands were quantified by densitometric scanning. The specificity of the gel shift complexes was determined by preincubation with a 70-fold excess of unlabeled NF- κ B probe, or nonspecific unlabeled activator protein (AP-1) oligonucleotide. To identify the subunits that constitute the NF- κ B complex present in nasal tissue samples, specific antisera that recognize the p50, p65, and c-Rel subunits were preincubated with the nuclear extracts before the addition of the labeled probe.

To standardize the expression of NF- κ B activity, we used as an internal standard the EMSA values of 19 healthy nasal mucosa (14 men; mean age 36 ± 9 years) undergoing nasal corrective surgery for turbinate hypertrophy or septal dysmorphism. We gave the value of 100 to the mean value of NF- κ B densitometric scanning measurements obtained from healthy nasal mucosa, and we expressed the amount of NF- κ B binding of each sample as the percentage change with respect to this control.

Data analysis

mRNA data is reported as the arithmetic mean $\pm$ standard error of the mean (SEM) of 10^6 molecules of COX-2 RNA/ μ g of total RNA. Nonparametric statistical analysis was performed using the Kruskal–Wallis test and the Mann–Whitney *U*-test for between-group comparisons. Pearson's correlation was used to assess the relationship between COX-2 expression and NF- κ B activity. A *P* value < 0.05 was regarded as statistically significant.

Results

COX-2 mRNA

The mean levels of COX-2 mRNA expression in nasal polyps from the AIAR group (0.25 ± 0.06) were significantly lower than in polyps from the ATAR group (1.58 ± 0.50 , $P < 0.01$) (Fig. 1).

The COX-2 mRNA levels in polyps from ATAR patients who were on corticosteroid therapy (1.38 ± 0.2)

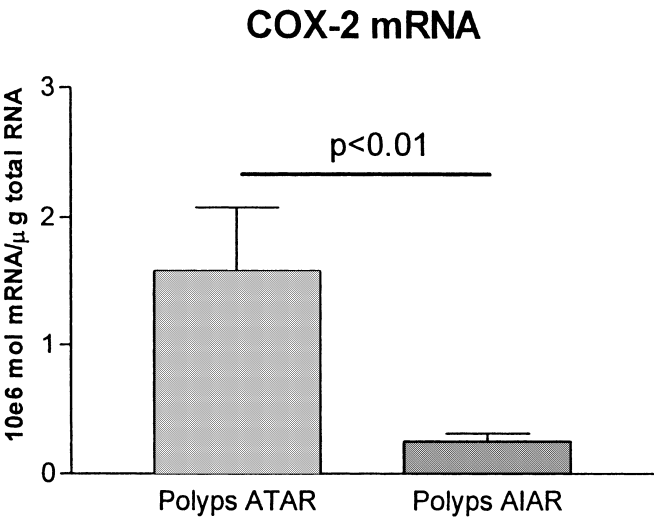


Figure 1. Expression of cyclooxygenase-2 (COX-2) mRNA in nasal polyps from aspirin-tolerant and aspirin-intolerant asthma/rhinitis patients (ATAR and AIAR groups, respectively). There was a significant difference between ATAR and AIAR.

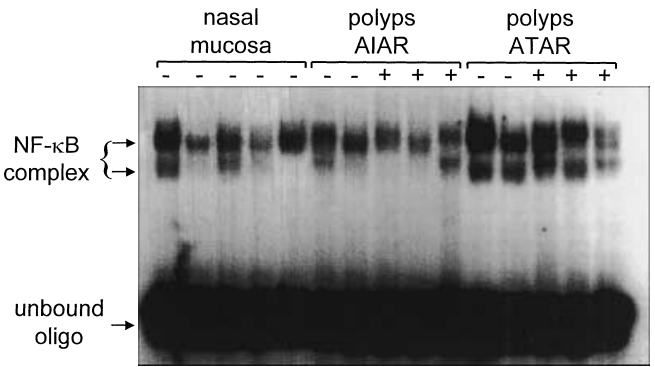


Figure 2. Representative electrophoretic mobility shift assay showing specific nuclear factor(NF)-κB-binding activity, as demonstrated by the intensity of the retarded band (arrow), in five nasal mucosa (lanes 1–5), five nasal polyps from aspirin-hypersensitive asthma/rhinitis patients (AIAR; lanes 6–10), and five nasal polyps from lanes 11–15). Patients on corticosteroid therapy (+) and patients not on corticosteroid therapy (–) before polypectomy. Corticosteroids had not significant effects on NF-κB activity (see Results). The radioactivity at the bottom of the gel is free probe (unbound oligonucleotide).

at the time when polypectomy was performed were similar to the levels from patients who had discontinued corticosteroid therapy before surgery (1.2 ± 0.6). Similar results were found in polyps from AIAR patients (0.31 ± 0.1 vs. 0.25 ± 0.1).

NF-κB electrophoretic mobility shift assay (EMSA)

NF-κB binding activity was measured by EMSA in nuclear extracts from nasal mucosa and nasal polyps (Fig. 2).

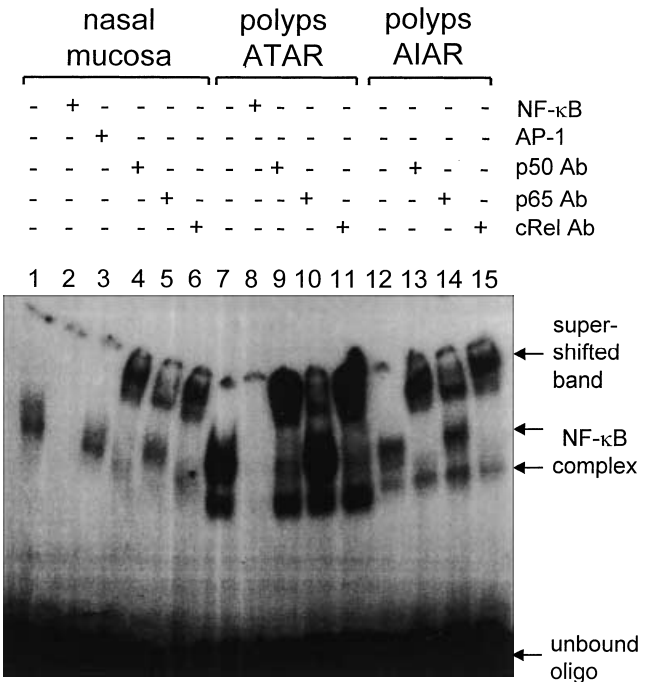


Figure 3. Electrophoretic mobility shift assay of the nuclear extracts from six nasal mucosa (lanes 1–6), five nasal polyps from aspirin-tolerant asthma/rhinitis patients (ATAR; lanes 7–11) and four nasal polyps from aspirin-hypersensitive asthma/rhinitis patients (AIAR; lanes 12–15). Specificity of the binding was confirmed by addition of a 70-fold excess of unlabeled nuclear factor(NF)-κB oligonucleotide (lanes 2 and 8) or excess noncompetitive unlabeled activator protein-1 oligonucleotide (lane 3). Supershift assays were performed with 4 μg of nuclear extracts, which were incubated with 20 000 counts per minute of ^{32} P-labeled consensus oligonucleotide for NF-κB binding in the absence (lanes 1, 7, 12) or presence of antisera directed against p50 (lanes 4, 9, 13), p65 (lanes 5, 10, 14), or c-Rel (lanes 6, 11, 15). The radioactivity at the bottom of the gel is free probe (unbound oligonucleotide).

Both types of tissue contained one retarded band, which was absent in the presence of an excess of unlabeled NF-κB probe, but not in the presence of an unrelated oligonucleotide (AP-1) (Fig. 3). Analysis of the subunit composition by means of supershift assay showed that specific antisera directed against the p50, p65 and c-Rel subunits supershifted the NF-κB retarded bands in nasal mucosa and nasal polyps from both ASA-tolerant and ASA-hypersensitive patients (Fig. 3). These observations indicate that nasal mucosa and nasal polyps have the same pattern of subunit composition.

Densitometric scanning measurement of the NF-κB-retarded bands corresponding to nasal mucosa and nasal polyps from ATAR and AIAR are shown in Fig. 4. Values are expressed as percentage of change of densitometric scanning measurements with respect to the control (mean value of all nasal mucosa). NF-κB activity was

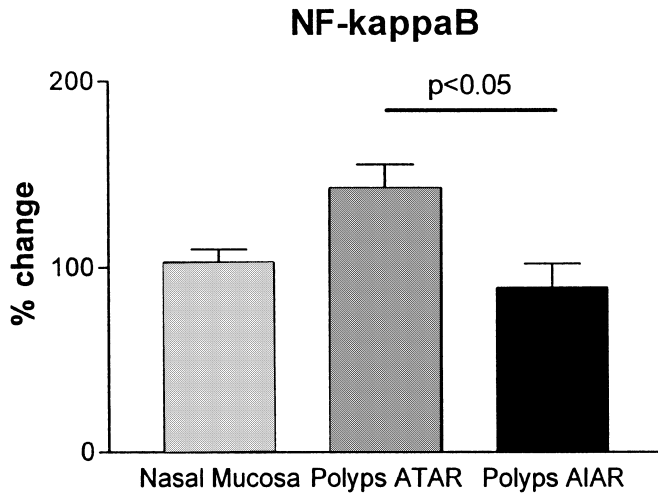


Figure 4. Densitometric scanning measurement of the nuclear factor(NF)- κ B retarded bands corresponding to nasal mucosa ($n = 19$), aspirin-tolerant asthma/rhinitis (ATAR) nasal polyps ($n = 17$), and aspirin-intolerant asthma/rhinitis (AIAR) polyps ($n = 23$). We gave the value of 100 to the mean value of NF- κ B densitometric scanning measurements obtained from nasal mucosa (internal standard control). The amount of NF- κ B-binding of each sample is expressed as percentage with respect to this control.

significantly ($P < 0.05$) higher in nasal polyps from ATAR (143 ± 12), compared to nasal polyps from AIAR (89 ± 13). Although, NF- κ B activity in nasal mucosa was also higher than in AIAR polyps, the difference did not reach significant levels.

The NF- κ B activity in polyps from patients who were receiving corticosteroid therapy (145 ± 20) did not statistically differ from those who were not in treatment (141 ± 15) before polypectomy.

There was no correlation between COX-2 mRNA expression and NF- κ B activity ($r = 0.23$).

Discussion

Aspirin and other NSAIDs cause bronchoconstriction in about 5–10% of asthmatic people (1). Although it is not yet clear why some asthmatics are hypersensitive to aspirin, it seems most likely that aspirin-induced asthma is in some way secondary to the capacity of all NSAIDs to inhibit the COX pathway (1).

There is accumulated evidence to suggest that aspirin hypersensitivity may be due to an abnormal COX metabolism in aspirin-sensitive people, even in the absence of aspirin challenge (3–7, 11, 18). Sczeklik et al. (3) reported an increase in susceptibility of nasal polyps from AIAR patients to the inhibitory action of aspirin. Schmid et al. (5) and Yamashita et al. (7) found low levels of prostaglandins in nasal polyps from AIAR

compared to ATAR patients. Kowalski et al. (4) also reported that cultured epithelial cells obtained from polyps of AIAR patients generated less PGE₂ than similar cells obtained from ATAR patients. Finally, Schäfer et al. (18) found a significantly lower release of PGE₂ in peripheral blood cells from the AIAR group than the ATAR group.

In keeping with previous studies, we found the presence of activated NF- κ B in nasal mucosa and nasal polyps (17). Supershift analysis of the retarded bands also shows the presence of p50, p65, and c-Rel of NF- κ B in nasal mucosa and nasal polyps.

Our results show for the first time a decrease in expression of NF- κ B, as measured with the NF- κ B-DNA binding assay in nasal polyp specimens from patients with aspirin hypersensitivity. The results also show low expression of COX-2 in the same polyps—a finding reported previously by our group (11). Several reports have shown that activation of NF- κ B triggers the expression of COX-2 gene; our findings suggest that deficient activation and/or nuclear transition of NF- κ B in nasal polyps from AIAR may account at least in part for down-regulation of COX-2 expression in nasal polyps from these patients.

The lack of correlation between NF- κ B activity and COX-2 expression measured at an isolated point can be explained by differences in the kinetics of both substances. Although an interdependent regulation exists between the transcriptional factor and the enzyme, a time lag after NF- κ B gene binding and increased COX-2 mRNA expression usually occurs.

The signaling cascade leading to COX-2 up-regulation also involves the participation of some transcription factors other than NF- κ B, such as NF-IL6 and C/EBP β (13, 19). Strong evidence indicates that interactions between NF- κ B and other transcription factors influence the ability of NF- κ B to regulate gene expression in a selective manner (14). The lack of correlation between NF- κ B activity and COX-2 mRNA expression may also reflect the complexities of the mechanism that regulates inducible genes.

The mechanism which causes dysregulation of NF- κ B and possibly contributes to down-regulation of COX-2 in AIAR patients is unclear. Typically, intense rhinitis usually precedes bronchial asthma and aspirin hypersensitivity (1). The clinical course of aspirin-sensitive asthma has been used to support the hypothesis that a persistent infection may be the stimulus that induces the whole process (1). Recent studies have demonstrated that noninvasive variants of fungal species are frequently detected in the sinonasal region in people with chronic rhinosinusitis and nasal polyposis (20, 21). Interestingly, the fungus *Aspergillus fumigatus*, and other pathogenic fungi, synthesize a substance called gliotoxin which is a potent inhibitor of NF- κ B (22). The possible involvement of chronic fungus colonization in the down-regulation of NF- κ B in some patients with chronic

rhinosinuitis and nasal polyps needs to be explored in further studies.

In this study we also found that corticosteroid therapy had no effects on activity of either COX-2 or NF- κ B in nasal polyps. These findings agree with previous *in vivo* studies reporting that corticosteroid treatment does not result in any change (11), or can even increase the expression of COX-2 mRNA (23). Similarly, *in vivo* (24) and *in vitro* studies (25–27), have reported either no effect or an enhanced effect of corticosteroids on NF- κ B DNA-binding activity.

In summary, we have used nasal mucosa and nasal polyps to show that NF- κ B is down-regulated in AIAR patients. Since NF- κ B activation induces COX-2 mRNA

expression and COX-2 is down-regulated in AIAR patients, our results suggest that dysregulation of the transcriptional factor may account at least in part for low expression of COX-2 and eventual decreases in the production of PGE₂ in nasal polyps of aspirin-sensitive patients.

Acknowledgments

This study was supported by grants from: Fondo de Investigaciones Sanitarias (00/0969), Sociedad Española de Neumología y Cirugía Torácica (SEPAR), and Generalitat de Catalunya (2000SGR/120).

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