Original articles

Enhanced expression of cyclo-oxygenase isoenzyme 2 (COX-2) in asthmatic airways and its cellular distribution in aspirin-sensitive asthma

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Abstract
Background – There are two isoforms of cyclo-oxygenase (COX), namely COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and in blood platelets. The metabolites derived from COX-1 are probably involved in cellular housekeeping functions. COX-2 is expressed only following cellular activation by inflammatory stimuli and is thought to be involved in inflammation.
Methods – The expression of COX-1 and COX-2 isoenzymes has been studied in the bronchial mucosa of 10 normal and 18 asthmatic subjects, 11 of whom had aspirin-sensitive asthma (ASA) and seven had non-aspirin-sensitive asthma (NASA).
Results – There was a significant fourfold and 14-fold increase, respectively, in the epithelial and submucosal cellular expression of COX-2, but not of COX-1, in asthmatic patients. There was no significant difference in the total number of cells staining for either COX-1 or COX-2 between subjects with ASA and NASA, but the number and percentage of mast cells that expressed COX-2 was significantly increased sixfold and twofold, respectively, in individuals with ASA. There was a mean fourfold increase in the percentage of COX-2 expressing cells that were mast cells in subjects with ASA and the number of eosinophils expressing COX-2 was increased 2.5-fold in these subjects.
Conclusions – COX-2-derived metabolites may play an essential part in the inflammatory processes present in asthmatic airways and development of drugs targeted at this isoenzyme may have therapeutic potential in the treatment of asthma. Mast cells and eosinophils may also have a central role in the pathology of aspirin-sensitive asthma.

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Keywords: cyclo-oxygenase, aspirin-sensitive asthma, mast cells, epithelium.

The cyclo-oxygenase (COX) pathway converts arachidonic acid to prostaglandins, prostacyclin, and thromboxane A_2.[1 2] There are two isoforms of COX, namely COX-1 and COX-2.[3] COX-1 (PGH synthase-1) was initially purified and cloned from sheep vesicular glands and is constitutively expressed in most tissues and in blood platelets. The metabolites derived from COX-1 are probably involved in cellular housekeeping functions such as coordinating the actions of circulating hormones and regulating vascular homeostasis. COX-2 (PGH synthase-2) shares approximately 62% amino acid homology with COX-1[3] and is expressed only following cellular activation by inflammatory stimuli such as growth factors, cytokines and mitogens in fibroblasts and by bacterial lipopolysaccharide in monocytes and macrophages.[4–7] The observations that the expression of COX-2 is induced by inflammatory mediators in immune cells and that its expression is inhibited by glucocorticoids suggest that inducible COX-2 may produce prostanoids involved in inflammation. Since enhanced cytokine expression is a feature of asthmatic airways, we reasoned that COX-2 might be augmented in this disease.

Patients with bronchial asthma form a heterogeneous group and asthmatic individuals with aspirin sensitivity are a distinct clinical subpopulation. These patients have accompanying symptoms of rhinosinusitis including rhinorrhoea, nasal congestion, anosmia, loss of taste, and recurrent severe nasal polyposis.[8] Upon ingestion of aspirin, cysteinyl leukotrienes are released probably from eosinophils and mast cells and this is accompanied by worsening of asthmatic symptoms.[9–14] These symptoms do not occur in aspirin-tolerant subjects. Accumulating evidence suggests that COX inhibition is central to the mechanism for enhanced cysteinyl leukotriene synthesis[13 15–17] but the pattern of COX-1 and COX-2 expression in aspirin-sensitive asthma is unknown. We have therefore evaluated the expression of COX isoenzymes in asthmatic airways of individuals with and without aspirin sensitivity.

Table 1 Clinical characteristics of aspirin-sensitive subjects

Subject no.	Sex	Age (years)	Drugs	FEV_1 (l)	FEV_1 (% predicted)	Lysine-asprin (mg/ml)	Skin test	Eosinophils ($\times 10^3/\mu l$)
1	M	57	nBDP (200 µg)	3.76	104	25	−ve	0.35
2	F	56	BUD (300 µg), A, T (700 mg), IB	2.10	91	2.5	−ve	0.39
3	M	39	BDP (1500 µg), A, T (700 mg), nBUD (200 µg)	4.11	109	2.5	+ve	1.01
4	M	56	BDP, A (4 mg), IB, F	3.52	104	7.5	+ve	0.34
5	M	54	PRED (2 mg), BUD (800 µg), A, T (600 mg)	3.2	93	2.5	+ve	0.36
6	F	26	BDP (400 µg), A	2.93	87	25	+ve	0.32
7	F	33	PRED (6 mg) BUD (1200 µg), A, T (700 mg)	3.8	119	12.5	+ve	1.79
8	M	56	BDP (1000 µg) nBUD (250 µg), A, IB	3.54	82	25	−ve	0.29
9	M	40	BUD (600 µg), A, T(400 mg)	2.42	71	10	+ve	0.95
10	F	26	BUD (300 µg), A (800 µg)	3.58	99	5	−ve	0.27
11	M	24	BUD (800 µg), nBUD (200 µg), A	3.08	81	1.25	−ve	0.32
Mean (SE)		42.5 (4.14)		3.28 (0.18)	94.55 (4.23)	7.50*		0.49 (0.15)

Lysine-asprin = threshold dose of lysine-aspirin producing a 20% fall in FEV_1; PRED = prednisolone; BDP = beclomethasone dipropionate; BUD = budesonide; n = nasal route; A = albuterol; T = theophylline; IB = ipratropium bromide; C = cromolyn; F = fenoterol; R = reproterol.
* Geometric mean.

Table 2 Clinical characteristics of non-asprin sensitive subjects

Subject no.	Sex	Age (years)	Drugs	FEV_1 (l)	FEV_1 (% predicted)	Skin test	Eosinophils ($\times 10^3/\mu l$)
1	F	53	BDP (200 µg, C, R (10 mg)	3.40	133	−ve	0.14
2	M	35	C, R (10 mg)	4.57	102	+ve	0.41
3	M	24	A, T* (875 mg), C (10 mg)	3.68	82	+ve	0.29
4	M	25	IB, F	3.93	95	+ve	0.92
5	M	25	BUD (600 µg), IB, F	3.52	78	+ve	0.08
6	M	30	BDP (200 µg), A	2.28	52	+ve	0.46
7	F	37	PRED (5 mg), BUD (300 µg), nBUD (200 µg), A, T (850 mg)	1.59	54	+ve	0.19
Mean (SE)		34 (3.6)		3.28 (0.38)	85.1 (10.7)		0.36 (0.11)

For definitions of abbreviations see footnote to table 1.

Methods

SUBJECTS

Ten normal volunteers (seven men) of mean (SE) age 23 (1) years and 18 asthmatic patients (12 men) of mean (SE) 39 (3) years with forced expiratory volume in one second (FEV_1) of 90.9 (4.8)% were studied, of whom 11 had aspirin-sensitive asthma (ASA) and the remainder had non-aspirin-sensitive asthma (NASA). The diagnosis of aspirin sensitivity was made on the basis of history and confirmed by a 20% or greater fall in FEV_1 following lysine aspirin inhalation challenge as previously described.[14] The NASA group also underwent lysine aspirin challenge and, if negative, this was confirmed by incremental challenge with 600 mg of aspirin given orally.

The ASA subjects (seven men, six atopic) had a mean (SE) age of 43 (4) years with a mean FEV_1 of 94.6 (4.2)% and the seven NASA subjects (five men, six atopic) had a mean age of 34 (4) years with a mean FEV_1 of 85.1 (10.7)%. Of the 11 subjects with ASA two were taking prednisolone orally, 10 were taking regularly inhaled corticosteroids, and four were using regular nasal corticosteroids (table 1). Of the seven subjects with NASA one was using prednisolone orally, four were using regular inhaled corticosteroids, and one

subject was taking regular nasal corticosteroids (table 2). All asthmatic subjects were using inhaled or oral β_2 agonists as required. All subjects underwent skin prick tests to common aeroallergens and atopy was defined as the presence of at least two positive reactions (weals of 3 mm or more compared with saline control) to cat fur, dog hair, grass pollen, and *Dermatophagoides pteronyssinus*.

Bronchoscopic examination was performed and biopsy samples of the airway mucosa were taken as previously described[14] at least one month after aspirin provocation. Bronchial biopsy samples were immediately snap frozen in embedding medium and stored at −70°C until analysed. All subjects provided written and informed consent and the study was approved by the Hochgesbirgsklinik, Davos-Wolfgang ethics committee.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed as previously described[14] using the avidin biotin complex (ABC) technique for single immunostaining and the ABC technique combined with the indirect alkaline phosphatase technique for the double immunostaining. Affinity purified rabbit polyclonal anti-COX-1 or anti-COX-2
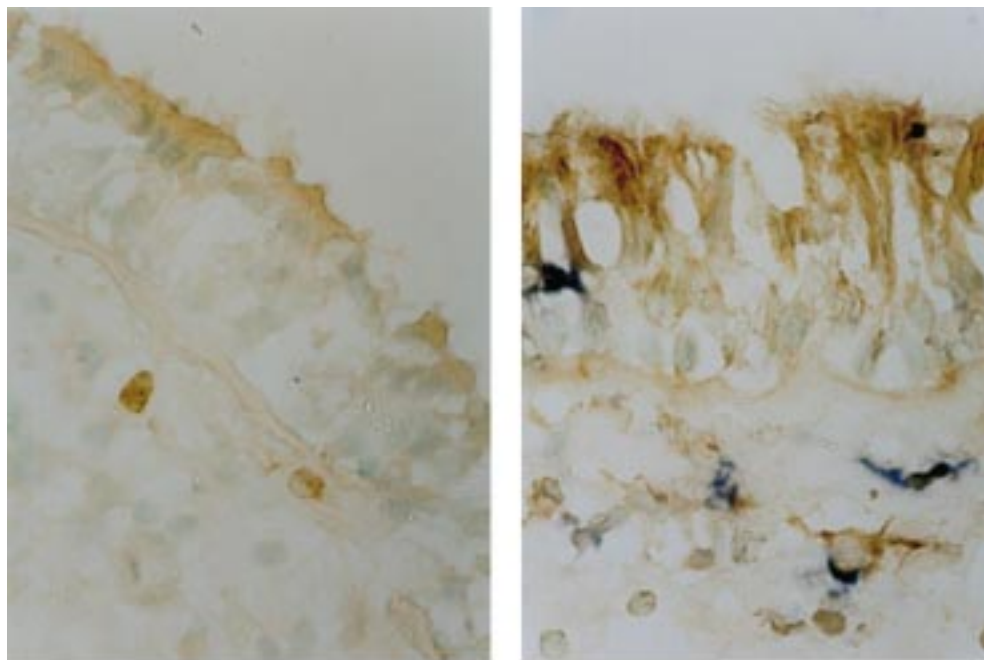
Figure 1   (A) Asthmatic airway stained with COX-1 polyclonal antibody. The photograph shows minimal staining of the airway epithelium and two inflammatory cells which have stained for COX-1. (B) Asthmatic airway stained with COX-2 polyclonal antibody. The photograph shows significant staining of airway epithelial cells and submucosal inflammatory cells. The submucosal cells have been double stained for COX-2 and for macrophages with a monoclonal antibody to macrophages (EBM11).

(gift from Dr Jilly Evans, Merck Frosst, Canada) were used at a dilution of 1/1000. All the primary antibodies used for cell identification were mouse monoclonal IgG_1 antibodies and comprised the following: AA1 (DAKO, High Wycombe, UK), a mast cell tryptase marker used at a dilution of 1/50; EBM11 (DAKO), a pan-macrophage marker that recognises CD68 used at 1/100 dilution; anti-CD3 (DAKO), a pan-T lymphocyte marker used at 1/50 dilution; NP57 (DAKO), an anti-human neutrophil elastase antibody used at 1/600 dilution; BMK13 (Cymbus Bioscience Ltd, Chilworth, UK), a pan-eosinophil marker that recognises major basic protein used at 1/40 dilution. The secondary antibodies were a goat anti-mouse alkaline phosphatase conjugated antibody (Sera-lab, Crawley Down, UK) used at a dilution of 1/100 and a swine anti-rabbit biotinylated antibody (DAKO) used at a dilution of 1/200. Single staining was performed on the frozen bronchial biopsy specimens with the rabbit polyclonal antibody to COX-1.

The biopsies were double immunostained using a rabbit polyclonal antibody to COX-2 concurrently with one monoclonal antibody to each cell type: neutrophils (NP57); macrophages (EBM11); eosinophils (BMK13); mast cells (AA1); and T lymphocytes (anti-CD3). Staining for COX-1 was by the ABC technique as previously described[14] with the modification that incubation with the two secondary antibodies were made separately and for 30 minutes each. Endogenous biotin, alkaline phosphatase and peroxidase activity was abolished as previously described.[14] The immunoperoxidase colour reaction was developed by incubation with diaminobenzidine, which produces a brown precipitate, and 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium (Sigma, Poole, Dorset, UK) which produces a blue precipitate. A positive control using nasal polyp tissue and a negative control without primary antibody was included in each staining run.

IMAGE ANALYSIS

The hue-saturation-intensity method of colour image analysis was adopted for detection of the brown immunoperoxidase reaction product, as previously described.[18] The total epithelial area in each biopsy specimen was measured.

The numbers of specific cells were counted on the entire submucosal biopsy area by a blinded investigator. The total section area was measured using the image analyser and all cell counts were expressed as cells /mm².

STATISTICAL ANALYSIS

Statistical analysis of the COX-1 and COX-2 data was performed by the Mann-Whitney U test, using Minitab Software (Minitab Inc.) Results are expressed as mean (SE).

Results

EXPRESSION OF COX-1 AND COX-2 IN
BRONCHIAL EPITHELIUM

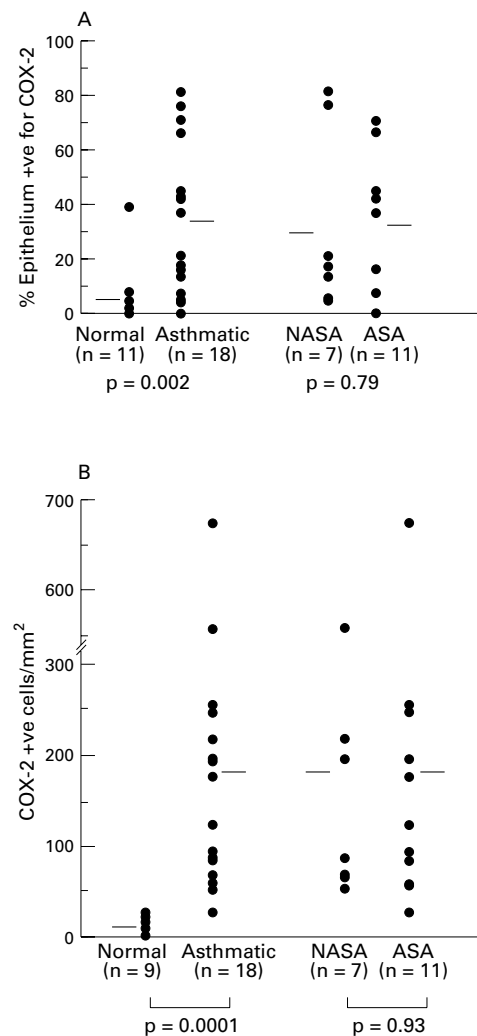There was no significant difference in the epithelial expression of COX-1 between normal

Figure 2 Expression of COX-2 in (A) the bronchial epithelium in normal asthmatic non-aspirin-sensitive (NASA) and aspirin-sensitive (ASA) asthmatic subjects and (B) the numbers of COX-2-staining cells in the airway in normal asthmatic NASA and ASA subjects.

shows an example of an asthmatic airway stained for COX-1. In contrast, there was a mean fourfold increase in the bronchial epithelial expression of COX-2 in asthmatic subjects (p=0.002; fig 2A), but there was no significant difference in the bronchial epithelial expression of COX-2 between subjects with ASA and NASA (p=0.79). Figure 1B shows an example of an asthmatic airway stained for COX-2.

EXPRESSION OF COX-1 AND COX-2 IN BRONCHIAL SUBMUCOSA
There was no significant difference between the number of COX-1 positive cells in the submucosa of normal and asthmatic subjects (90.4 (18.3) cells/mm² and 118.4 (20.4) cells/mm², respectively, p=0.56) or between subjects with ASA and NASA (97.5 (23.0) cells/mm² and 148.3 (36.2) cells/mm², respectively, p=0.31). In contrast, there was a significant mean 14-fold increase in the number of COX-2 positive cells in the submucosa from asthmatic subjects (p=0.0001; fig 2B), but there was no significant difference between the total number of COX-2 positive cells in the bronchial submucosa from subjects with ASA and NASA (180.9 (54.5) cells/mm² and 178.3 (67.9) cells/mm², respectively, p=0.93).

CELLULAR PROFILE IN BRONCHIAL SUBMUCOSA OF SUBJECTS WITH ASA AND NASA
There were more mast cells (AA1) (79.0 (14.6)/mm² versus 28.25 (7.06/mm²; p=0.01) and more total eosinophils (BMK13) (157.0 (30.9)/mm² versus 49.77 (7.31)/mm²; p=0.01) in the bronchial submucosa of subjects with ASA compared with NASA. There were no significant differences in bronchial biopsy cell numbers for activated eosinophils (EG2) (100.0 (28.3) versus 56.0 (12.9); p=0.25), neutrophils (NP57) (104.3 (29.2)/mm² versus 100.72 (9.9)/mm²; p=0.36), total macrophages (EBM11) (239.5 (62.5)/mm² versus 207 (62.5)/mm²; p=0.79), or T lymphocytes (CD3) (56.1 (22.9)/mm² versus 74.6 (35.0)/mm²; p=0.59) between subjects with ASA and NASA.

and asthmatic subjects (2.4 (0.7)% and 1.6 (0.4)%, respectively, p=0.23) or between subjects with ASA and NASA (1.3 (0.5)% and 2.3 (0.7)%, respectively, p=0.53). Figure 1A
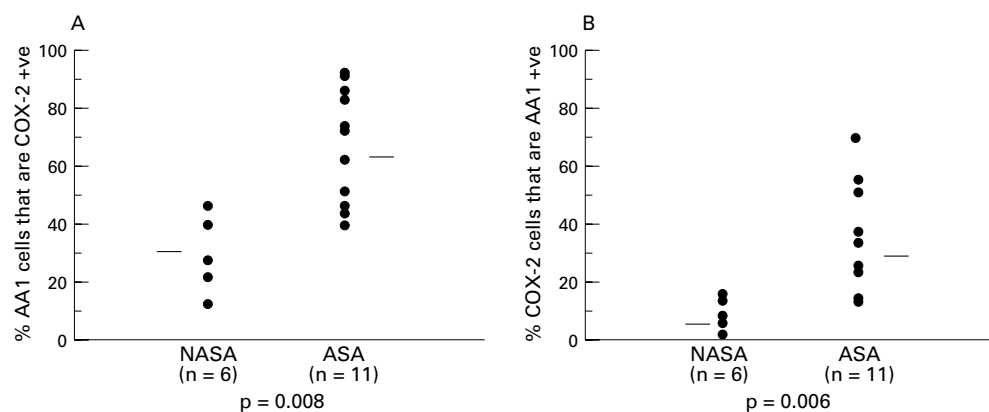

Figure 3   (A) Percentage of mast cells (identified using the anti-tryptase antibody AA1) expressing COX-2 immunostaining and (B) percentage of COX-2-containing cells that are mast cells.
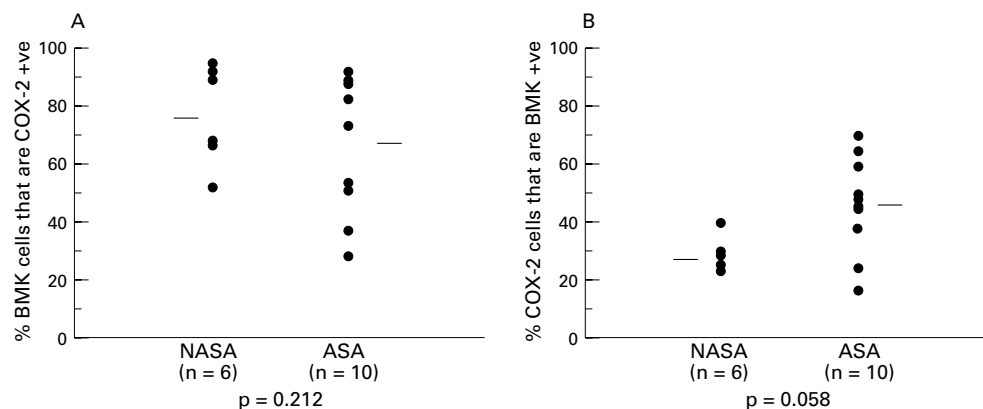
Figure 4 (A) Percentage of eosinophils (identified using the anti-major basic protein marker BMK13) expressing COX-2 and (B) percentage of COX-2-staining cells that are eosinophils.

SUBMUCOSAL CELLULAR EXPRESSION OF COX-2

There was a significant mean sixfold increase in the numbers of mast cells expressing COX-2 (p=0.004) between subjects with ASA and NASA (56.2 (12.7) cells/mm^2 and 9.0 (2.0) cells/mm^2, respectively). There was also a significant mean twofold increase in the percentage of mast cells expressing COX-2 (p=0.008) and a mean fourfold increase in the percentage of COX-2-expressing cells that were mast cells (p=0.006) in subjects with ASA compared with NASA (fig 3A and B).

There was a significant mean 2.5-fold increase in the number of total eosinophils expressing COX-2 (p=0.044) between subjects with ASA and NASA (104.8 (29.7) cells/mm^2 and 39.9 (7.9) cells/mm^2, respectively). The percentage of total eosinophils expressing COX-2 and the percentage of COX-2 cells that were eosinophils was also raised in subjects with ASA but the difference failed to reach statistical significance (p=0.212 and p=0.058, respectively; fig 4A and B).

There was no significant difference in the expression of COX-2 in activated eosinophils, neutrophils and macrophages of subjects with ASA and NASA. T lymphocytes did not stain for COX-2.

Discussion

COX exists in at least two isoforms with similar molecular weights (approximately 70 kDa).[19] COX-1 is involved in maintaining normal physiological function of tissues – for example, it produces prostacyclin which when released by the endothelium is antithrombogenic[20] and when released by the gastric mucosa is cytoprotective.[21] COX-2 expression is induced by pro-inflammatory cytokines in vitro in cell types such as endothelial cells, macrophages, and fibroblasts[7 22 23] and in vivo its expression is augmented in inflamed lesions.[19] Non-steroidal anti-inflammatory drugs inhibit the activity of COX, a property which explains both its therapeutic and adverse effect. The ability of non-steroidal anti-inflammatory drugs to inhibit COX-2 may explain their therapeutic potential as anti-inflammatory drugs, whereas inhibition of COX-1 may account for their unwanted side

effects such as gastric and renal damage. The two COX isoenzymes are pharmacologically distinct.[7] For example, aspirin completely inhibits bis-oxygenation of arachidonate by COX-1, while aspirin treated COX-2 metabolises arachidonate primarily to 15-lipoxygenase-derived metabolites such as 15-hydroxy-eicosatetraenoic acid (15-HETE), instead of PGH$_2$, due to acetylation of COX-2 by aspirin.[24] 15-HETE could affect the function of any of a number of cells and tissues involved in homeostasis and inflammation. Aspirin, indomethacin, and piroxicam are more potent at inhibiting COX-1, whereas flurbiprofen, meclofenamate, and diclofenac are equipotent at inhibiting COX-1 and COX-2.

There are no previous reports of the expression of COX-1 and COX-2 isoenzymes in bronchial asthma. We have shown that asthmatic bronchial epithelium and submucosal inflammatory cells have enhanced expression of COX-2, suggesting that the quantities and profile of COX-2-derived metabolites may be important in the amplification of the inflammatory response in asthmatic airways. The results were obtained by comparing biopsy samples obtained by fibreoptic bronchoscopy from asthmatic and normal control individuals. The mucosal biopsy samples were analysed using specific antibodies to each of the COX isoenzymes, as well as to individual cell types. The use of double immunohistochemistry allowed co-localisation of cyclo-oxygenase isoenzyme to specific cells. The levels of COX-1, the non-inducible isoform, did not increase in asthma, indicating that the inflammatory milieu in bronchial asthma does not affect its regulation. Previous clinical observations that non-steroidal anti-inflammatory drugs such as indomethacin and aspirin do not benefit the majority of asthmatic patients have suggested that COX metabolites have little part to play in the mechanisms of airways inflammation. However, the discovery of the inducible isoform of COX now demands a reinterpretation of these clinical observations, especially since the airway microenvironment in asthma with the abundance of pro-inflammatory cytokines will encourage the enhanced expression of COX-

2. Specific COX-2 inhibitors may have a therapeutic use in asthma.

Aspirin intolerance is marked by the development of bronchospasm, naso-ocular symptoms, urticaria, angio-oedema, and anaphylaxis after ingestion of non-steroidal anti-inflammatory drugs. These symptoms may occur in isolation or in any combination.[8] The prevalence of aspirin sensitivity in asthma is estimated to be 10–30%. Symptoms generally appear after the third decade of life and are frequently associated with chronic rhinitis, sinusitis, and nasal polyposis. These subjects often have persistent asthma, requiring regular treatment with either inhaled or oral corticosteroids.[25 26] The propensity for non-steroidal anti-inflammatory drugs to precipitate reactions in aspirin-sensitive individuals is related to the capacity of these drugs to inhibit COX.[7 19] Previous work has shown that aspirin treated COX-2 metabolises arachidonic acid primarily to 15-lipoxygenase metabolites instead of PGH_2.[24] There were no significant differences in the age, baseline lung function, or the usage of medication between subjects with ASA and NASA. The diagnosis of aspirin sensitivity was confirmed by lysine-aspirin bronchoprovocation, whereas the control group of NASA individuals were proven to be aspirin-tolerant by both inhalation and oral aspirin challenge. We have confirmed in this study the findings of a previous study[14] that the numbers of mast cells and eosinophils are increased in the airways of ASA subjects. The present data extend these observations by demonstrating that there is an increase in the numbers of mast cells and eosinophils expressing COX-2 in subjects with ASA. The percentage of mast cells staining for COX-2 and the percentage of COX-2-containing cells that are mast cells are also increased in patients with ASA. These data support the view that these two cell types may participate in enhancing the basal airways inflammation in asthmatic patients with aspirin sensitivity. However, it is unlikely that the increased expression of COX-2 in the mast cell and eosinophil contributes to the mechanism of the aspirin-provoked asthmatic response, since administration of nimesulide (100 mg), a COX-2 inhibitor, to aspirin-sensitive asthmatic patients did not provoke bronchoconstriction.[27]

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