

# Eicosanoid mediator expression in mononuclear and polymorphonuclear cells in normal subjects and patients with atopic asthma and cystic fibrosis

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## Abstract

**Background** – Eicosanoids such as leukotrienes, prostaglandins, lipoxins, and 15-hydroperoxyeicosatetraenoic acid (15-HETE) cause bronchoconstriction, increased microvascular permeability, mucus secretion, and polymorph chemotaxis. These pro-inflammatory effects are important in diseases such as asthma and cystic fibrosis where the levels of mediators are increased both in the stable and acute state. A study was conducted to examine the expression of the mRNA for the enzymes of the eicosanoid pathways (5-lipoxygenase (5-LO), 5-lipoxygenase activating protein (FLAP), cyclo-oxygenases 1 and 2 (COX-1, COX-2), and 15-lipoxygenase (15-LO)) in normal subjects and in patients with stable atopic asthma and stable cystic fibrosis.

**Methods** – Reverse transcription polymerase chain reaction (RT-PCR) was used to examine the expression of total RNA for 5-LO, FLAP, COX-1, COX-2, and 15-LO in peripheral blood polymorphonuclear cells and mononuclear cells from the three subject groups.

**Results** – The expression of mRNA for 5-LO and FLAP was similar in normal subjects and in patients with asthma and cystic fibrosis. COX-1 was increased in both cell types in asthmatic patients. COX-2 and 15-LO were increased in polymorphs of patients with atopic asthma but not in mononuclear cells. COX-2 and 15-LO were undetectable in either cell type in patients with cystic fibrosis whereas COX-1 levels in polymorphs were similar to those in patients with asthma.

**Conclusions** – The increased leukotriene production in asthma and cystic fibrosis is not explained by an increase in transcription of 5-LO and FLAP. Transcription of 15-LO and COX-2 is increased in atopic asthma. Transcription of COX-1 is increased in both atopic asthma and cystic fibrosis.

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**Keywords:** asthma, cystic fibrosis, eicosanoids, leukotrienes.

The eicosanoids, a group of mediators implicated in the inflammatory response, are the product of the metabolism of arachidonic acid by three

separate enzymes. The enzyme 5-lipoxygenase (5-LO), together with 5-lipoxygenase activating protein (FLAP), results in the formation of the leukotrienes; the two isoforms of cyclo-oxygenase, COX-1 and COX-2, in the formation of prostaglandins, prostacyclin and thromboxane; and 15-lipoxygenase (15-LO) in the formation of 15-hydroperoxyeicosatetraenoic acid (15-HETE) and the lipoxins. The leukotrienes are pro-inflammatory, causing polymorphonuclear cell chemotaxis, bronchoconstriction, increased mucus secretion, and increased vascular permeability.<sup>1,2</sup> Individual prostaglandins have opposing activities. Prostaglandins formed by COX-1 are responsible for the maintenance of cellular function whereas the inducible COX-2 is responsible for production of the pro-inflammatory prostaglandins.<sup>3,4</sup> The products of 15-LO are interesting in that they are anti-inflammatory. They antagonise the effects of the leukotrienes, inhibit free radical production, and inhibit receptor-mediated cellular activation.<sup>5-8</sup>

Eicosanoids are important in inflammatory diseases such as asthma and cystic fibrosis. Increased levels of leukotrienes have been demonstrated in both stable asthma and during acute exacerbations,<sup>9-11</sup> and both the prostaglandins and lipoxin A<sub>4</sub> have been found in a number of pulmonary diseases including asthma.<sup>9,12</sup> Furthermore, inhibition of leukotriene production improves asthma.<sup>13</sup>

Despite the evidence of increased eicosanoid mediator levels in asthma and cystic fibrosis, to our knowledge there has been no investigation into the regulation of eicosanoid production in these patient groups. We have used a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) approach to examine the expression of the mRNA for 5-LO and FLAP, COX-1, COX-2, and 15-LO in peripheral blood mononuclear cells and polymorphonuclear cells of normal subjects and patients with stable atopic asthma and stable cystic fibrosis.

## Methods

### MATERIALS

All chemicals were of molecular biology grade and purchased from Sigma (Poole, UK). Ficoll Paque was purchased from Pharmacia (St Albans, UK). RT-PCR components were purchased from Promega except for Taq DNA polymerase (Boehringer Mannheim, Lewes, UK). Primers were synthesised by R & D Systems, Abingdon, UK.

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Primer	Sequence	Expected size
GAPDH	F 5'-TTC CAT GGC ACC GTC AAG GCT R 5'-TCA GGT CCA CCA CTG ACA CGT T	468
COX-1	F 5'-TGC CCA GCT GGC CCG CCG CTT R 5'-GTG CAT CAA CAC AGG CGC CTC TTC	301
COX-2	F 5'-TTC AAA TGA GAT TGT GGG AAA ATT GCT R 5'-AGA TCA TCT CTG CCT GAG TAT CTT	304
5-LO	F 5'-ATC AGG ACG TTC ACG GCC GAG G R 5'-CCA GGA ACA GCT CGT TTT CCT G	300
FLAP	F 5'-GGC AAT GTT GTC CTG CTG TTG GCC R 5'-GCG TTC TCT CTC CTA GGT AAC	350
15-LO	F 5'-GTT CCA ACA ACC AGG TGC AGC R 5'-GCT CTT CTT CCC GGT GTT TCT	447

Figure 1 Primer sequences for the genes of interest. GAPDH = glyceraldehyde phosphate dehydrogenase; COX-1 = cyclo-oxygenase 1; COX-2 = cyclo-oxygenase 2; 5-LO = 5-lipoxygenase; FLAP = 5-lipoxygenase activating protein; 15-LO = 15-lipoxygenase.

#### PATIENTS

The study was approved by the ethics committee of the Royal Brompton, National Heart and Lung Hospitals. All subjects gave informed consent. Normal subjects were volunteers who gave a negative history for atopy and had a normal peripheral blood eosinophil count and total IgE count. Atopic asthmatic patients had asthma as defined by the American Thoracic Society and were stable on inhaled  $\beta_2$  agonists and inhaled corticosteroids in doses of less than 1200  $\mu\text{g}/\text{day}$ . All had a positive history for atopy (eczema or hayfever) and an elevated total IgE count. Some also had a mild peripheral blood eosinophilia. Patients with cystic fibrosis were attending the outpatients department of the London Chest Hospital; they had had no infective exacerbations for the previous six weeks and were taking no inhaled or oral corticosteroids. Their only other treatments were vitamin E and pancreatic enzyme supplements.

#### CELL SEPARATION

Fifty ml of venous blood was collected into a syringe containing 8 ml of acid citrate dextran (ACD), immediately placed on ice, and all subsequent steps performed at 4°C to minimise activation of the cells. The white blood cells were separated by density gradient centrifugation using Ficoll Paque. The resulting mononuclear cell and polymorphonuclear cell layers were removed and washed with 2–4 volumes of cold Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. Cells were resuspended in 1 ml of cold HBSS, an aliquot was removed, and cell numbers were counted and purity assessed on a haemocytometer. In all subsequent steps the preparations were more than 98% pure.

#### RNA ISOLATION

The RNA was extracted according to the method of Gough<sup>14</sup> in the presence of RNase

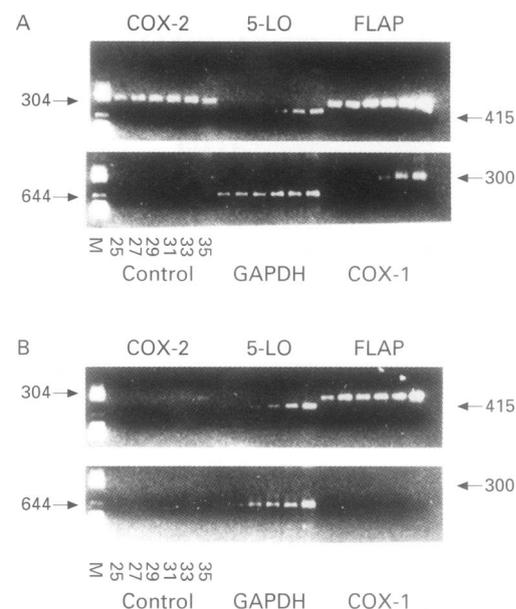


Figure 2 Detection of 1  $\mu\text{g}$  of mRNA by RT-PCR in normal and asthmatic subjects. (A) Analysis of PCR product detected after increasing cycles of PCR in polymorphonuclear cells from an atopic asthmatic subject. The result is representative of 15 experiments. (B) As for (A) but for a normal subject. The result is representative of eight experiments. 15-LO was negative for each of these subjects. M = DNA size markers.

inhibitors. The RNA was washed twice in ethanol and precipitated, and the amount of total RNA quantified by spectrophotometry at 260 nm (Uvikon 940 Spectrophotometer, Kontron, Zurich, Switzerland).

#### REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

One  $\mu\text{g}$  of total RNA was reverse transcribed into cDNA with AMV reverse transcriptase and oligo (dT)<sub>15</sub> primers according to standard protocols (Promega, Southampton, UK).

PCR was performed on the resulting cDNA using specific primers for COX-1, COX-2, 5-LO, FLAP, 15-LO, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (fig 1) using Taq polymerase according to the manufacturer's instructions. Up to 35 cycles were performed (95°C, 1 minute; 60°C, 1 minute; 72°C, 1 minute) for all primer sets except 15-LO (annealing temperature 63°C). GAPDH was used as a positive control for the PCR reaction and a no template cDNA control as the negative control. Aliquots (8  $\mu\text{l}$ ) were removed at 25, 27, 29, 31, 33 and 35 cycles, size fractionated on a 2% agarose gel, and the cycle number at which the correct product first appeared determined (fig 2A and B).

#### SEMI-QUANTITATION OF PCR PRODUCTS

The PCR products were cloned into pGEM5z vectors and the product sequenced to confirm identity. For further quantification linearised plasmid containing the PCR product was diluted to concentrations between  $4.5 \times 10^{-10}$  mol and  $2 \times 10^{-22}$  mol and PCR performed for the cycle number at which 50% of the samples

Table 1 Characteristics of subjects in the three study groups

	Sex (M:F)	Age range (years)	Atopy	Inhaled steroids	WCC ( $\times 10^9/l$ )	Eosinophils ( $\times 10^9/l$ )	IgE (IU/l)	FEV <sub>1</sub> (% predicted)
Normal subjects (n = 7)	7:0	26–38	No	No	5.99	0.08	61 (14–176)	105
Atopic asthma (n = 6)	3:3	28–64	Yes	Yes	6.40	0.188	219 (23–643)	103
Cystic fibrosis (n = 10)	8:2	24–30	1 yes/9 no	2 yes/8 no	13.65	0.216	53 (6–3339)	62

WCC = white cell count; FEV<sub>1</sub> = forced expiratory volume in one second.

were first positive in each group. The concentration of product was determined by the lowest concentration that produced a visible band. This gave a measure of the minimum amount of mRNA for each of the products assuming approximately equivalent reverse transcription prior to the PCR. For those groups where less than 50% of samples were positive at 35 cycles, PCR of the concentration series was performed for 35 cycles and the starting template estimated to be less than the lowest concentration visible on the gel at 35 cycles.

### Results

All subjects had a full blood count and serum IgE level performed at the same time that their blood was taken for RNA extraction. Group characteristics for age, sex, atopic status, inhaled corticosteroid use, blood counts, IgE level, and lung function are summarised in table 1.

The proportions of samples in each group positive for COX-1, COX-2, FLAP, and 15-LO remained constant with increasing cycle number. COX-1 mRNA was detected in polymorphs and mononuclear cells from normal subjects and from patients with asthma and cystic fibrosis. By 35 cycles there was an approximately threefold greater number of transcripts in polymorphs from both asthmatic (88%) and cystic fibrosis (75%) patients compared with normal subjects (33%) (fig 3A). In contrast, increased levels of COX-1 transcripts were seen only in the asthmatic patients when measured in mononuclear cells. COX-2 transcripts were not detected in polymorphs or mononuclear cells of any patients with cystic fibrosis (fig 3B). In comparison, COX-2 mRNA transcripts were detected in polymorphs from 20% of normal subjects and 50% of asthmatic subjects; 25% of normal subjects expressed COX-2 mRNA in mononuclear cells compared with undetectable levels in asthmatic patients.

By 27 cycles 5-LO mRNA was detected in approximately 30% of mononuclear cells and polymorphs from both normal and asthmatic subjects (fig 4A). This basal level of expression was much reduced in patients with cystic fibrosis in whom only 16% expressed 5-LO in mononuclear cells and none in polymorphs. At 25 cycles FLAP mRNA could be detected in more than 50% of mononuclear cells and polymorphs from both normal and asthmatic subjects but it was completely absent in both

cell types from patients with cystic fibrosis (fig 4B). There was no difference in FLAP mRNA expression between asthmatic and normal subjects in polymorphs (62%) but an increased level of FLAP mRNA was detected in mononuclear cells of asthmatic patients (75%) compared with normal subjects (40%).

By 33 cycles 15-LO was not detectable in any of the patients with cystic fibrosis in either cell type (fig 4C). Transcripts were detected in approximately 10% of mononuclear cells from normal and asthmatic subjects and in polymorphs from normal subjects. In contrast, there was a significant increase in 15-LO in the polymorphs isolated from asthmatic patients (75%).

When PCR analysis was continued to 35 cycles it was possible to detect mRNA in cells at lower concentrations (table 2). Products not detected at 35 cycles suggested that the cDNA sample contained less than 0.002 attomoles

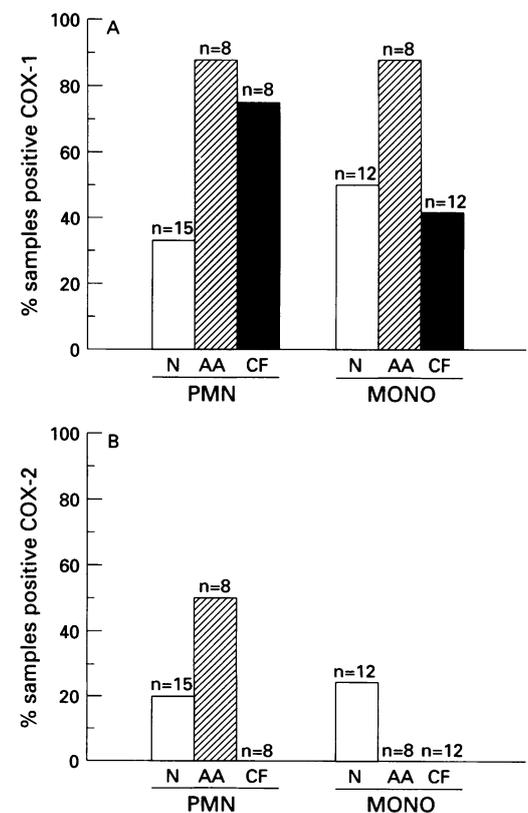


Figure 3 Comparison of the number of samples positive for (A) COX-1 and (B) COX-2 in normal subjects (N) and patients with atopic asthma (AA) and cystic fibrosis (CF) at 35 cycles. PMN = polymorphonuclear cells; MONO = mononuclear cells.

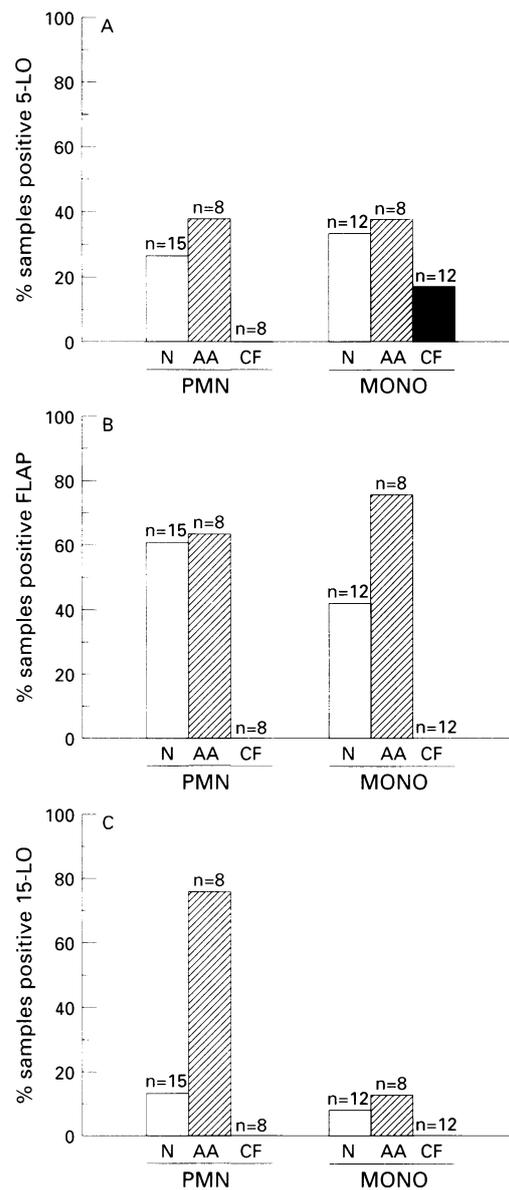


Figure 4 Comparison of the number of samples positive for (A) 5-LO at 27 cycles, (B) FLAP at 25 cycles, and (C) 15-LO at 33 cycles in normal subjects (N) and patients with atopic asthma (AA) and cystic fibrosis (CF). PMN = polymorphonuclear cells; MONO = mononuclear cells.

( $2 \times 10^{-21}$  mole or approximately 1500 copies of cDNA) which was less than one transcript per 10 000 cells and therefore considered as not present. After 35 cycles the level of 5-LO mRNA was greater in polymorphs from both asthmatic and cystic fibrosis patients compared with normal subjects, whereas there was no difference in 5-LO mRNA in mononuclear cells

Table 2 Cumulative percentage of samples positive by 35 cycles for COX-1, COX-2, 5-LO, FLAP, and 15-LO in normal subjects and patients with atopic asthma and cystic fibrosis in peripheral blood polymorphonuclear cells and mononuclear cells

	Polymorphonuclear cells			Mononuclear cells		
	Normal	Atopic asthma	Cystic fibrosis	Normal	Atopic asthma	Cystic fibrosis
COX-1	33	87.5	75	50	87.5	41.6
COX-2	20	50	0	25	0	0
5-LO	40	62.5	62.5	58	50	50
FLAP	93.3	75	87.5	91.6	100	100
15-LO	40	75	0	25	25	0

between any of the groups. FLAP mRNA was detected in most samples from polymorphs and mononuclear cells in all the subject groups. There was increased expression (75%) of 15-LO mRNA in polymorphs from asthmatic subjects compared with normal subjects (40%). This difference was not present in mononuclear cells. 15-LO was not detected in either polymorphs or mononuclear cells from patients with cystic fibrosis.

Semi-quantitation of each of the cDNA products confirmed that detection at higher cycle numbers corresponded to less starting template (table 3). All transcripts were less abundant than GAPDH, with 5-LO and FLAP being 10–100 times less and COX-1, COX-2, and 15-LO 100 000 to one million times less abundant. The amounts of 5-LO and FLAP were similar in all three groups, and 15-LO was 1000 times more abundant in polymorphs from asthmatic subjects than in any other group. COX-1 transcripts were more abundant in asthmatic subjects than in normal subjects or patients with cystic fibrosis, and the number of COX-2 transcripts was marginally greater in polymorphs from asthmatic subjects than in other groups.

## Discussion

In this study we examined the basal expression of the mRNA for the enzymes involved in the production of eicosanoid mediators in normal subjects and compared this with two diseases characterised by chronic airway inflammation. We were able to detect increased transcripts of COX-1 mRNA in polymorphonuclear cells and in mononuclear cells of atopic asthmatics when compared with normal subjects. COX-2, in contrast, was increased only in polymorphs from asthmatic subjects when compared with normal subjects and patients with cystic fibrosis. 5-LO transcripts were similar in all three groups, as were FLAP mRNA transcripts. 15-LO mRNA transcripts were increased in the polymorph fraction of patients with atopic asthma.

A semi-quantitative approach to PCR using known concentrations of our PCR products was used to enable a comparison between normal subjects and patients with two inflammatory diseases. More accurate quantitation to the level of determining copies of mRNA per cell was possible but would have been extremely time consuming with the number of samples generated. The semi-quantitation confirmed the results of the serial sampling in that the later appearance of product correlated with lower concentrations of starting cDNA template and therefore less mRNA. Examination of the enzymes involved in leukotriene production showed that the mRNA for both 5-LO and FLAP was abundant. There was no difference between the 5-LO and FLAP mRNA levels seen in normal subjects and patients with asthma, therefore increased transcription does not explain the increased production of leukotrienes in asthmatic patients. This suggests that control of leukotriene production may be post-transcriptional. In contrast, during differ-

Table 3 Relative concentrations of starting cDNA template for GAPDH, COX-1, COX-2, 5-LO, FLAP, and 15-LO, based on a concentration series of the cloned product, in normal subjects and patients with atopic asthma and cystic fibrosis in peripheral blood polymorphonuclear and mononuclear cells

	Polymorphonuclear cells			Mononuclear cells		
	Normal	Atopic asthma	Cystic fibrosis	Normal	Atopic asthma	Cystic fibrosis
GAPDH	5 ( $\times 10^{-17}$ )	2.5 ( $\times 10^{-16}$ )	2.5 ( $\times 10^{-17}$ )	5 ( $\times 10^{-17}$ )	2.5 ( $\times 10^{-16}$ )	2.5 ( $\times 10^{-18}$ )
COX-1	<5 ( $\times 10^{-22}$ )	2.5 ( $\times 10^{-21}$ )	5 ( $\times 10^{-22}$ )	5 ( $\times 10^{-22}$ )	2.5 ( $\times 10^{-20}$ )	<5 ( $\times 10^{-22}$ )
COX-2	<2.6 ( $\times 10^{-22}$ )	2.6 ( $\times 10^{-22}$ )	<2.6 ( $\times 10^{-22}$ )			
5-LO	<2 ( $\times 10^{-18}$ )	2 ( $\times 10^{-18}$ )	2 ( $\times 10^{-18}$ )	2 ( $\times 10^{-18}$ )	2 ( $\times 10^{-17}$ )	2 ( $\times 10^{-18}$ )
FLAP	2 ( $\times 10^{-18}$ )	2 ( $\times 10^{-18}$ )	2 ( $\times 10^{-19}$ )	4.5 ( $\times 10^{-19}$ )	2 ( $\times 10^{-18}$ )	2 ( $\times 10^{-19}$ )
15-LO	<3 ( $\times 10^{-22}$ )	2 ( $\times 10^{-19}$ )	<3 ( $\times 10^{-22}$ )			

entiation of HL-60 cells by DMSO the amount of 5-LO transcript is increased but there is no post-translational modification of the enzyme, no increase in transcription following stimulation with calcium ionophore, and no loss of cytosolic enzyme following nuclear translocation.<sup>15,16</sup> If the enzyme can be “recycled” there would be less need for large amounts of its mRNA transcript. Evidence for this has been described in mast cells following antigenic stimuli.<sup>17</sup> The levels of 5-LO and FLAP mRNA transcripts we have found are in keeping with studies that suggest that the expression in peripheral blood cell monocytes is low and increases dramatically once they become macrophages, together with increased production of eicosanoids.<sup>18–20</sup> Migration of polymorphs to the inflammatory site and their activation may result in a similar upregulation of 5-LO and FLAP expression and increased production of leukotrienes.

In contrast, in patients with cystic fibrosis the mRNA for all the enzymes was detected later and reflects the slightly lower concentrations of the starting template. Similar results for 5-LO and FLAP were obtained, however. This is in contrast to the increased levels of leukotrienes found in the sputum of patients with cystic fibrosis who have high levels of leukotrienes even when “well” between acute infective exacerbations.<sup>21,22</sup> This again suggests post-transcriptional control of leukotriene production in peripheral blood cells in patients with cystic fibrosis.

There were clear differences between the expression of mRNA for COX-1 and COX-2 in mononuclear cells and polymorphs. As expected, the “constitutive” form of cyclo-oxygenase, COX-1, was detected in most samples. More samples were positive in the two disease groups than in the normal subjects. Although COX-1 levels are thought to be stable, several studies have suggested that serum stimulatory factors are capable of upregulating COX-1 expression, and this may explain the increased expression in these two inflammatory diseases.<sup>23</sup> In contrast, more COX-2 was detectable in the polymorphs of asthmatic patients, but no COX-2 was detected in patients with cystic fibrosis and it was found in only 20–25% of normal subjects. The lack of detectable COX-2 in all patients with asthma and cystic fibrosis was somewhat surprising, given the inflammatory nature of their illness, although these patients had been chosen while stable. Inhibitory feedback of further prostaglandin production or post-transcriptional con-

trol of prostaglandin production such as activation of protein kinase C to divert eicosanoid production may be responsible.<sup>24</sup> Alternatively, the absence of detectable COX-2 in peripheral blood cells may be due to their lack of activation or differentiation into macrophages, since tissue macrophages express more COX-2 and produce more prostaglandins.<sup>25</sup>

15-LO was detected in increased amounts in polymorphs from asthmatic patients. Eosinophil-enriched leucocyte preparations contain 15-LO whilst immunofluorescence studies have detected the protein only in eosinophils.<sup>26,27</sup> Thus, increased amounts detected in the polymorph fraction in our study in asthmatic subjects are likely to be due to eosinophils. Although our experimental technique did not allow us to determine the amount of 15-LO per million eosinophils, the 1000 times greater amount of 15-LO in polymorphs from asthmatic subjects is greater than would be expected given that the eosinophil count in normal subjects was  $0.08 \times 10^9/l$  and in asthmatic subjects was  $0.188 \times 10^9/l$ .

At present there is little information available on stimuli that regulate 15-LO expression. In peripheral blood mononuclear cells of normal volunteers the cytokines IL-4 and IL-13 upregulate 15-LO mRNA, enzyme and products, and hydrocortisone reduces it.<sup>28,29</sup> It has been suggested that 15-LO is an effector molecule for IL-4.<sup>30</sup> In addition, several immunohistochemistry studies have demonstrated 15-LO enzyme in normal, bronchitic and asthmatic airways, predominantly in airway epithelial cells and in eosinophils in the submucosa.<sup>27,31</sup> The greatest staining was seen in asthmatic airways. It is tempting to postulate that the increase in 15-LO in asthma is in response to the chronic inflammation – an attempt to limit the inflammation and aid resolution. In an experimental glomerulonephritis model elevated levels of 15-LO are associated with a reduction in pro-inflammatory mediators such as leukotrienes, and with resolution of the renal lesions.<sup>32</sup>

The absence of 15-LO in peripheral blood cells in cystic fibrosis is therefore puzzling. It is possible that there is a defect in the anti-inflammatory response leading to a loss of 15-LO. In bronchial epithelial cells from patients with cystic fibrosis there is evidence that the anti-inflammatory cytokine IL-10 is down-regulated.<sup>33</sup> However, given the abundant inflammatory response in the lung in cystic fibrosis, it is equally possible that 15-LO is present and upregulated, but limited to ac-

tivated cells such as the eosinophil and alveolar macrophage already in the lung. The peripheral blood cells sampled would therefore not be representative of activated cells, hence the lower levels of 15-LO expressed. Clearly, further work is needed to answer these questions.

The patients in this study were specifically chosen to be on low dose inhaled corticosteroids to minimise any effect they might have on the expression of these enzymes. The plasma level of corticosteroids in these patients is likely to be extremely low. While the effect of corticosteroids on 5-LO is controversial, and on 15-LO unknown, they do downregulate COX-2 expression.<sup>3</sup> An effect on COX-2 expression cannot be excluded and, if present, would underestimate the increase in COX-2 in these inflammatory diseases.

In summary, by using semi-quantitative PCR to compare basal levels of mRNA for the enzymes involved in eicosanoid production we have been able to demonstrate that there is little difference in 5-LO and FLAP expression between normal subjects and patients with atopic asthma and cystic fibrosis, whereas COX-1, COX-2 and 15-LO are increased in asthmatic patients and COX-2 and 15-LO were undetectable in patients with cystic fibrosis. This suggests that the level of control of eicosanoid production varies considerably between pathways and involves predominantly post-transcriptional mechanisms.

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