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# Effect of interleukin-1$\beta$, tumour necrosis factor-$\alpha$ and interferon-$\gamma$ on the induction of cyclo-oxygenase-2 in cultured human airway smooth muscle cells

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**1** Increased levels of several pro-inflammatory cytokines including interleukin-1$\beta$ (IL-1$\beta$) and tumour necrosis factor-$\alpha$ (TNF$\alpha$) have been found in bronchoalveolar lavage fluid from symptomatic asthmatic patients. IL-1$\beta$, TNF$\alpha$ and interferon-$\gamma$ (IFN$\gamma$) are known to stimulate a number of cells to produce inflammatory mediators such as prostaglandins. Although airway smooth muscle (ASM) is known to be a rich source of prostaglandins, the regulation of cyclo-oxygenase (COX) isoforms and prostanoid production by proinflammatory cytokines have not been studied in human airway smooth muscle.

**2** We studied the effects of IL-1$\beta$, TNF$\alpha$ and IFN$\gamma$ on the induction of two isoforms of cyclo-oxygenase and its relation to prostaglandin $E_2$ ($PGE_2$) release and COX activity (reflected by $PGE_2$ synthesis from exogenous arachidonic acid) in human cultured airway smooth muscle cells.

**3** IL-1$\beta$, but not TNF$\alpha$ or IFN$\gamma$, caused a time- and concentration-dependent enhancement in $PGE_2$ and other prostanoid (6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, thromboxane $B_2$ ($TXB_2$) and $PGD_2$) production, with $PGE_2$ and 6-keto-$PGF_{1\alpha}$ as the principal products. This stimulation was accompanied by a corresponding increase in COX activity.

**4** COX-2 protein measured by Western blot analysis was not detectable in untreated cells, but was increased in a time- and concentration-dependent manner by IL-1$\beta$, but not TNF$\alpha$ or IFN$\gamma$. In contrast, no variation in the expression of COX-1 protein was observed.

**5** Pretreatment with the conventional non-steroidal anti-inflammatory drugs (NSAIDs), indomethacin and ibuprofen, and the selective COX-2 inhibitors, NS-398 and nimesulide, completely blocked IL-1$\beta$-induced $PGE_2$ release and COX activity. The glucocorticosteroid dexamethasone and protein synthesis inhibitors, cycloheximide and actinomycin D, not only markedly inhibited IL-1$\beta$-stimulated $PGE_2$ release and COX activity but also suppressed IL-1$\beta$-induced COX-2 induction.

**6** This study demonstrates that human cultured ASM cells release prostanoids in response to IL-1$\beta$ stimulation and that the response is mostly mediated by the induction of COX-2 rather than COX-1 isoenzyme, implying that airway smooth muscle may be an important source of prostaglandins in human airways and that COX-2 may play an important role in the regulation of the inflammatory process in asthma.

**Keywords:** Prostaglandin $E_2$; prostanoids; airway smooth muscle; interleukin-1$\beta$; tumour necrosis factor-$\alpha$; interferon-$\gamma$; cyclo-oxygenase; cyclo-oxygenase inhibitors; protein synthesis inhibitors; dexamethasone

## Introduction

The two main features of the pathogenesis of asthma are persistent airway inflammation and bronchial hyperresponsiveness. Prostanoids are important endogenous mediators involved in diverse biological processes such as cell proliferation, inflammation and immune responses. The effect of prostanoids on the airway may be either pro- or anti-inflammatory depending on the cell type, the balance of the prostanoids released and the prostanoid receptor subtype activated. Prostaglandin $E_2$ ($PGE_2$) is predominantly a protective prostaglandin (Knox & Tattersfield, 1995; Pavord & Tattersfield, 1995) whereas $PGD_2$, $PGF_{2\alpha}$ and thromboxane (TX) $A_2$ are pro-inflammatory mediators (Iwamoto *et al*., 1995; Johnston *et al*., 1995; Aizawa *et al*., 1996). The role of $PGI_2$ has been less well defined.

Cyclo-oxygenase (COX) is the enzyme at the rate-limiting step for the conversion of arachidonic acid (AA) to prostaglandins, prostacyclin ($PGI_2$) and $TXA_2$. COX exists in two isoforms (Xie *et al*., 1991). COX-1 is a constituent of healthy cells and is expressed under normal conditions. It is responsible for the production of prostaglandins under physiological conditions (Vane, 1994) and is important in circumstances where prostaglandins have a protective function, such as gastric mucus production and renal blood flow maintenance. COX-2, the inducible isoform of the enzyme, is the major isoenzyme associated with inflammation. COX-2 is induced in many cells by stimuli such as lipopolysaccharide (LPS; Hempel *et al*., 1994; Pang & Hoult, 1995; Pang *et al*., 1996) and pro-inflammatory cytokines (Jackson *et al*., 1993; Mitchell *et al*., 1994; Endo *et al*., 1995), whereas the expression of COX-1 usually remains unchanged. Increasing evidence suggests that the induction and regulation of COX-2 may be key elements in the pathophysiological process of a number of inflammatory disorders. Many studies have shown that bronchoalveolar lavage fluid from patients with symptomatic asthma contains significantly elevated levels of a number of pro-inflammatory cytokines including interleukin-1$\beta$ (IL-1$\beta$) and tumour necrosis factor-$\alpha$ (TNF$\alpha$) (Mattoli *et al*., 1991; Broide *et al*., 1992), which are known inducers of COX-2. Human alveolar macrophages, lung fibroblasts and airway epithelial cells have been shown to express COX-2 after stimulation with cytokines (mainly IL-1$\beta$) and LPS (Jackson *et al*., 1993; Mitchell *et al*., 1994; Hempel *et al*., 1994; Endo *et al*., 1995). Very recently, Asano and colleagues have shown that the phorbol ester phorbol 12-myristate 13-acetate (PMA) could induce COX-2 in a human cultured bronchial smooth muscle cell line (Asano *et al*., 1996), and COX-2 mRNA expression by IL-1$\beta$ and TNF$\alpha$ in rat tracheobronchial smooth muscle cells has also been demonstrated (Vadas *et al*., 1996). However, systematic studies are

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needed in the understanding of COX isoenzymes and prostanoid production in human airway smooth muscle (ASM) cells, to which cytokines could have a relatively easy access in asthma because the airway epithelium is damaged (Laitinen *et al*., 1985). As previous studies in bovine ASM have shown that ASM is an important source of $PGE_2$ (Delamere *et al*., 1994; Barry *et al*., 1995), it is of particular importance to study COX induction in ASM cells. In the present study, we have investigated the spectrum of prostanoid production by human ASM cells, the COX isoenzyme(s) responsible for this production under resting conditions and whether COX isoenzyme induction occurs in cells exposed to the inflammatory cytokines IL-1$\beta$, TNF$\alpha$ and interleukin-$\gamma$ (INF$\gamma$). In addition, we assessed the effect of non-selective COX inhibitors, selective COX-2 inhibitors, the protein synthesis inhibitors, cycloheximide and actinomycin D, and the anti-inflammatory steroid, dexamethasone, on $PGE_2$ release, COX activity and the expression of COX-1 and COX-2 enzyme proteins.

## Methods

### *Cell culture*

Primary cultures of human ASM cells were prepared from explants of airway smooth muscle according to the method described by Hall and co-workers (Hall *et al*., 1992; Widdop *et al*., 1993; Green *et al*., 1995) with some modifications. Human trachea was obtained from *post-mortem* individuals within 12 h of death. No patients had evidence of airway diseases as determined by history and pathological examination of the trachea and lungs. The tissue was transported to the laboratory in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), penicillin G (100 u $ml^{-1}$), streptomycin (100 $\mu$g $ml^{-1}$), amphotericin B (2.5 $\mu$g $ml^{-1}$) and L-glutamine (4 mM). It was then washed several times in the same medium. The trachealis muscle was then dissected free of epithelium and connective tissue under sterile conditions. Small ($2 \times 2$ mm) explants of airway smooth muscle were then excised and about 10 explants placed in small petri dishes. After explants were allowed to adhere, DMEM, containing FCS, antibiotics, amphotericin B and L-glutamine, was added to cover the explants. The explants were incubated in humidified 5% $CO_2$/95% air at 37°C and the medium was changed every 3 days. Smooth muscle cells were usually seen about 7 days later. When cells were about to become confluent in some parts of the petri dish, the explants were removed. Once confluent, cells were trypsinized with 0.25% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS), centrifuged and resuspended in the above medium, counted and plated out in several 75 $cm^2$ flasks and grown to confluence. Cells were then detached with trypsin-EDTA, resuspended in 90% FCS + 10% dimethyl sulphoxide at a density of $10^6$ cells $ml^{-1}$, frozen in liquid nitrogen and stored until required. Cells were thawed before use and plated at a density of $2 \times 10^4$ cells/well in 12-well culture plates containing the above medium.

### *Characterization of the ASM cells*

We employed morphological and immunocytochemical staining techniques to determine whether the cultured cells had the characteristics of pure ASM cells. Under the light microscope, subconfluent human ASM cells were spindle-shaped with central oval nuclei, while the confluent cells depicted the 'hill and valley' appearance which is characteristic of smooth muscle cells in culture (Chamley-Campbell *et al*., 1979; Campbell & Campbell, 1993). For the identification of the markers of ASM cell phenotype, the cells were plated in 8-well chamber slide system, grown to confluence, growth arrested for 24 h and fixed with methanol. The cells were then examined by standard immunocytochemical techniques by use of polyclonal antibodies against smooth muscle cell specific $\alpha$-actin and mature muscle cell specific desmin. We demonstrated that >95% of the cultured cells were positively labelled. In contrast, staining for the epithelial marker cytokeratin was negative.

### *$PGE_2$ assay*

All experiments were performed in confluent cultures which were usually obtained on the seventh or eighth day of culture. The medium was then changed to serum-free and the cells were growth arrested for 24 h. Immediately before each experiment, the medium was changed again to fresh serum-free medium and the reagent to be tested was added. At indicated time intervals, the culture media were transferred to separate microcentrifuge tubes and centrifuged at 4,000 r.p.m. for 10 min. The supernatants were removed and stored at −20°C until the determination of $PGE_2$ content by radioimmunoassay (RIA) as described previously (Delamere *et al*., 1994; Barry *et al*., 1995). Briefly, duplicate 100 $\mu$l aliquots of medium were mixed with [$^3$H]-$PGE_2$ and then incubated with anti-$PGE_2$ antiserum at 4°C for 24 . The bound label-antibody complexes were separated by use of dextran coated charcoal to precipitate unbound label, and the radioactivity was determined. The sensitivity for $PGE_2$ was 41.9 pg $ml^{-1}$. The anti-$PGE_2$ antiserum did not discriminate between $PGE_1$ and $PGE_2$. The cross-reactivity determined in our assay was 2.6% with $PGE_{2\alpha}$, 6.1% with $TXB_2$, <2.5% with 6-keto-$PGE_{1\alpha}$ and $PGD_2$.

### *Cyclo-oxygenase assay*

COX activity was assayed functionally by washing the cells three times in PBS after they had been treated with IL-1$\beta$ for a certain period of time and then adding arachidonic acid (AA, final concentration 5 $\mu$M) for a further 30 min incubation in serum-free medium. The assessment of the effect of NSAIDs on COX activity was performed with the inhibitors present at the time of analysis. These samples were subjected to radioimmunoassay for $PGE_2$ and the resulting $PGE_2$ level from exogenous AA was taken as an index of COX activity.

### *6-Keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, $TXB_2$ and $PGD_2$ assay*

6-Keto-$PGF_{1\alpha}$ (a stable product of $PGI_2$), $PGF_{2\alpha}$ and $TXB_2$ (a stable product of $TXA_2$) were measured with enzyme immunoassay kits. $PGD_2$ release was measured with a $PGD_2$ $^3$H assay system. The assays were carried out according to the protocols of the kit manufacturers. The sensitivities of the assays were: 1.4, 0.78, 8.0 and 3.1 pg $ml^{-1}$, respectively. The cross-reactivity determined was as follows: the anti-6-keto $PGF_{1\alpha}$ serum had 0.54% cross-reactivity with $PGE_2$, 1.67 with $PGE_{2\alpha}$, 0.6% with $PGD_2$ and <0.5% with $TXB_2$; the anti-$PGE_{2\alpha}$ serum had 1.23% cross-reactivity with $PGE_2$, 0.6% with $PGD_2$ and <0.5% with 6-keto-$PGF_{1\alpha}$ and $TXB_2$; the anti-$TXB_2$ serum had cross-reactivity of 0.58% with $PGF_{2\alpha}$ and <0.5% with $PGE_2$, 6-keto-$PGF_{1\alpha}$ and $PGD_2$; the anti-$PGD_2$ serum had <0.5% cross-reactivity with all other prostanoids tested.

### *MTT assay for cell viability*

The toxicity of cytokines, COX inhibitors, protein synthesis inhibitors, dexamethasone and drug vehicle (1% DMSO) to human ASM cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Pang *et al*., 1996). After 24 h incubation with the chemicals, 20 $\mu$l of 5 mg $ml^{-1}$ MTT (thiazolyl blue) was added to the culture medium in 96 well plates and incubated for 1 h at 37°C. After removing the medium, 200 $\mu$l dimethyl sulphoxide (DMSO) was added to solubilize the blue coloured tetrazolium and the plates were shaken for 5 min and the $OD_{550}$ values were read in a microplate reader. Viability was set at 100% in control cells.

### *Western blot analysis*

Identification of COX-2 by Western blotting was performed by culturing human ASM cells in 12-well plates. After washing the cells with PBS, they were incubated for 5 min with an extraction buffer (0.9% NaCl, 20 mM Tris-HCl, pH 7.6, 0.1% triton X-100, 1 mM phenylmethylsulphonyl fluoride, 0.01% leupeptin) with gentle shaking. The cell extract was centrifuged (4000 *g*, 4°C, 10 min), and the protein concentration in the supernatant was determined with the Bio-Rad protein assay reagent. Sufficient aliquots of sample (30 $\mu$g protein per track) were mixed 1 : 1 with sample buffer (20 mM Tris-HCl, pH 6.8, 20% glycerol, 2% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol and 0.025% bromophenol blue) and boiled for 5 min prior to electrophoresis in $20 \times 20$ cm 7.5% SDS-polyacrylamide gel. Separated proteins were electroblotted to pure nitrocellulose membranes and the blot was blocked overnight at 4°C in PBS-T (PBS pH 7.4 with 0.3% Tween-20) containing 8% fat-free dried milk powder. The blot was then incubated with primary anti-COX-2 antibody (1 : 1000 in PBS-T containing 8% fat-free dried milk powder) for 2 h at room temperature. The blot was subsequently washed with PBS-T and incubated with secondary antibody (goat anti-mouse IgG linked to horseradish peroxidase conjugate, diluted 1 : 2000 in PBS-T-8% milk powder) for 1 h at room temperature. Semi-quantitative staining was achieved by using chemiluminescence detection; the blot was washed with PBS-T and then incubated with SuperSignal CL-HRP Substrate System for 1 min and finally exposed to Hyperfilm-ECL. Reprobing of COX-1 was carried out by incubating the membrane in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl) at 50°C for 30 min with occasional agitation, washing the membrane in a large volume of PBS-T, blocking the membrane overnight in PBS-T-8% milk powder at 4°C and then following the steps to perform immunodetection with anti-COX-1 antibody as described above.

### *Statistical analysis*

Data are expressed as mean and s.e.mean from *n* determinations. Statistical analysis was performed by using the statistical software SPSS (Norusis, 1995). Unpaired two-tailed *t* test or one way analysis of variance were used to determine the significance of differences between means. The results were adjusted for multiple testing by use of Bonferroni's correction. *P* values of less than 0.05 were accepted as statistically significant.

### *Materials*

Recombinant human interleukin-1$\beta$, $PGE_2$ indomethacin, ibuprofen, dexamethasone, cycloheximide, actinomycin D, sodium dodecyl sulphate (SDS), leupeptin, phenylmethylsulphonyl fluoride, DMEM, penicillin and streptomycin, L-glutamine, amphotericin B, insulin, transferrin, ascorbic acid, triton X-100, glycerol, $\beta$-mercaptoethanol, anti-smooth muscle $\alpha$-actin serum and anti-$PGE_2$ serum were all purchased from Sigma (Poole, Dorset, U.K.); recombinant human TNF-$\alpha$ and recombinant human IFN-$\gamma$ from Genzyme (West Malling, Kent, U.K.); [5,6,8,11,12,14,15(n)-$^3$H]-$PGE_2$ (6.737 TBq $mmol^{-1}$), Rainbow coloured protein molecular weight markers, $PGD_2$ $^3$H assay system and Hyperfilm-ECL from Amersham Life Science (Little Chalfont, Bucks, U.K.); pure nitrocellulose blotting membrane from Gelman Sciences (Northampton, Northants, U.K.); SuperSignal CL-HRP Substrate System from Pierce (Rockford, IL, U.S.A.); polyclonal anti-mouse IgG coupled with horseradish peroxidase from Transduction Laboratories (Lexington, KY, USA); selective COX-2 inhibitors, NS-398 (N-(2-cyclohexyloxy-4-nitrophe

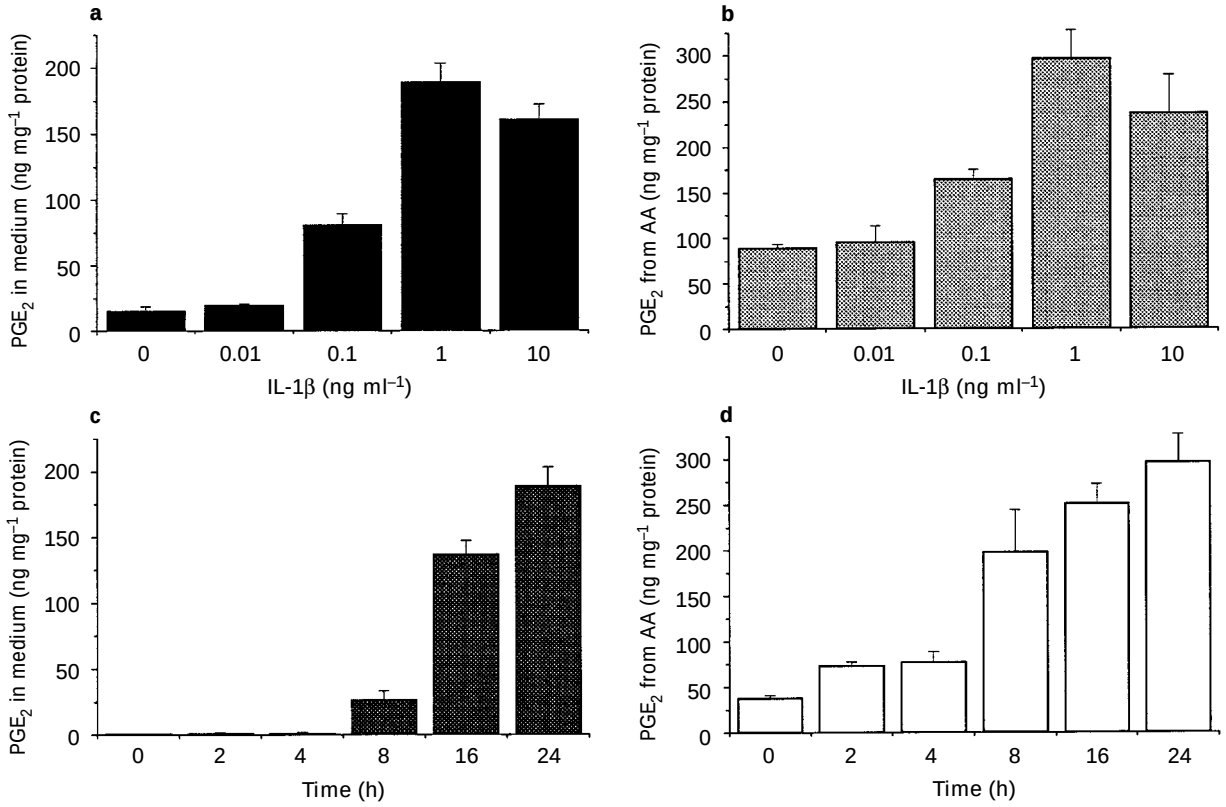


**Figure 1** Concentration-response and time course of the effect of interleukin-1$\beta$ (IL-1$\beta$) on $PGE_2$ production (a and c) and cyclooxygenase (COX) activity (b and d). Confluent human airway smooth muscle (ASM) cells in 12-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with various concentrations of IL-1$\beta$ for 24 h for the concentration response or with IL-1$\beta$ 1.0 ng $ml^{-1}$ for the times indicated. $PGE_2$ production was measured by RIA and COX activity was determined by measuring $PGE_2$ production from exogenous AA (5 $\mu$M, 30 min). Results are mean $\pm$ s.e.mean for a representative experiment ($n = 4$).

nyl)-methanesulphonamide) and nimesulide, monoclonal anti-human COX-2 antibody and monoclonal anti-ovine COX-1 antibody (with cross-reactivity to human COX-1) from Cayman Chemical (Ann Arbor, MI, U.S.A.); anti-desmin serum from Dako (High Wycombe, Bucks, U.K.); 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$ and $TXB_2$ enzyme immunoassay (EIA) kits from Cascade Biochem (Reading, Berks, U.K.).

## Results

### *$PGE_2$ release and COX activity in response to IL-1$\beta$*

Treatment of human ASM cells with recombinant human IL-1$\beta$ caused concentration-dependent and substantial release of $PGE_2$ ($P<0.001$) the maximal $PGE_2$ release occurring at 1 ng $ml^{-1}$ IL-1$\beta$ (Figure 1a). This was accompanied by induction of functionally active COX-2 as shown by washing the cells after 24 h exposure to IL-1$\beta$ followed by incubation with exogenous AA (Figure 1b). $PGE_2$ generation and COX activity from IL-1$\beta$ stimulated human ASM cells were also time-dependent (Figure 1c and d, $P<0.001$). Significant increases in COX activity could be seen as early as 2 h after stimulation. Direct evidence for time- and concentration-dependent induction in human ASM cells of COX-2 enzyme protein after exposure to IL-1$\beta$ was obtained by preparing Western blots of cell extracts with a specific antibody which resolves COX-2 from COX-1. As shown in Figure 2, untreated human ASM cells contained undetectable levels of COX-2. However, after exposure to IL-1$\beta$ there was a concentration- and time-related induction of COX-2 protein. Reprobing of the same blots with an antibody specific to COX-1 showed that COX-1 enzyme protein bands remained unchanged (Figure 2, lanes A–F and G–K). Incubation of human ASM cells with IL-1$\beta$ (0.01–10.0 ng $ml^{-1}$) did not show any effect on cell viability as measured by MTT assay (data not shown).

### *Release of other prostanoids as compared with $PGE_2$*

Untreated human ASM cells released low levels of all other prostanoids measured (6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, $TXB_2$ and $PGD_2$). However, incubation of the cells with IL-1$\beta$ release large amounts of 6-keto-$PGF_{1\alpha}$ (Figure 3) and caused significant accumulation of $PGF_{2\alpha}$, $TXB_2$ and $PGD_2$ (Figures 4 and 5) in a concentration- and time-dependent manner ($P<0.001$). The maximal concentrations of $PGF_{2\alpha}$, $TXB_2$ and $PGD_2$ were very low (2.67, 1.96, and 1.30 ng $mg^{-1}$ protein, respectively) as compared with that of $PGE_2$ (Figure 1c), while the maximal concentration of 6-keto-$PGF_{1\alpha}$ was 151 ng $mg^{-1}$ protein, close to that of $PGE_2$. When the results were plotted as % of $PGE_2$, it was interesting to note that as the time of stimulation went on, more $PGE_2$ was released into the medium, and the % of all these three low concentration prostanoids declined gradually with respect to the $PGE_2$ concentration (Figure 6), suggesting that $PGE_2$ became increasingly dominant in prostanoid generation in human ASM cells after IL-1$\beta$ stimulation. However, the ratio of 6-keto-$PGF_{1\alpha}$ and $PGE_2$ did not seem to have an obvious pattern (data not shown).

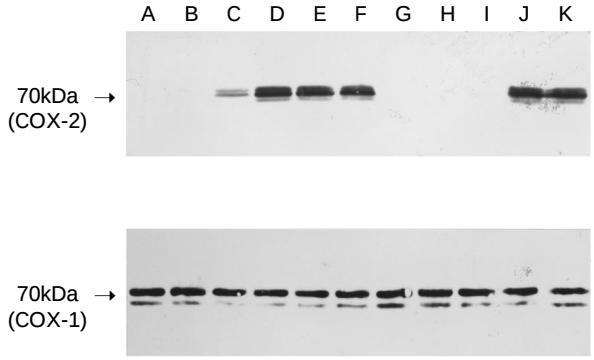


**Figure 2** Effect of interleukin-1$\beta$ (IL-1$\beta$) on the induction of cyclooxygenase-2 (COX-2) in human airway smooth muscle (ASM) cells. Western blot was performed by using specific antibodies to COX-2 and COX-1, respectively. Each lane was loaded with 30 $\mu$g of protein. Lanes A–F: 1.0 ng $ml^{-1}$ IL-1$\beta$ for 0, 2, 4, 8, 16 and 24 h; lane G: control cells cultured for 24 h, lanes H–K: 0.01, 0.1, 1.0 and 10.0 ng $ml^{-1}$ IL-1$\beta$ for 24 h. The positions and molecular weights of COX-1 and COX-2 were validated by reference to Rainbow coloured molecular weight markers. These blots are representative of similar results obtained at least three times.

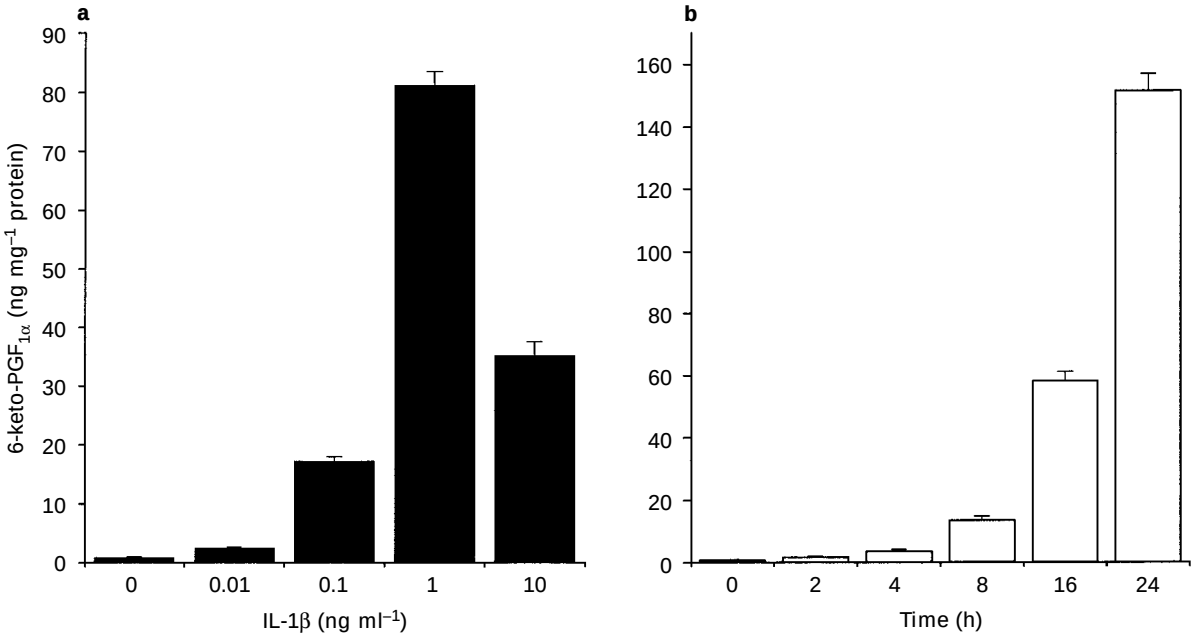


**Figure 3** Concentration-response (a) and time course (b) of the effect of interleukin-1$\beta$ (IL-1$\beta$) on production of 6-keto-$PGF_{1\alpha}$. Confluent human ASM cells in 12-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with various concentrations of IL-1$\beta$ for 24 h for the concentration-response or with IL-1$\beta$ 1.0 ng $ml^{-1}$ for the times indicated. 6-keto-$PGF_{1\alpha}$ production was measured by EIA. Results are mean$\pm$s.e.mean for a representative experiment ($n=4$).

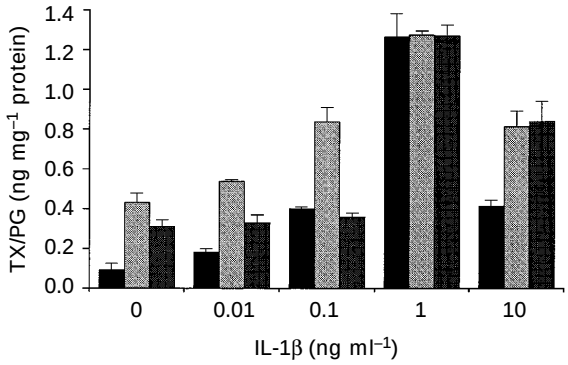


**Figure 4** Concentration-response effects of IL-1$\beta$ on $PGF_{2\alpha}$ (solid columns), $TXB_2$ (light stippled columns) and $PGD_2$ (dark stippled columns) release. Confluent human airway smooth muscle (ASM) cells in 12-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with various concentrations of IL-1$\beta$ for 24 h. $PGF_{2\alpha}$ and $TXB_2$ were determined by EIA and $PGD_2$ was measured by RIA. Results are mean$\pm$s.e.mean for a representative experiment ($n=4$).

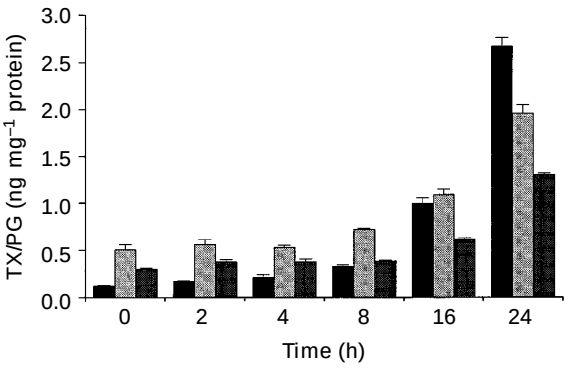


**Figure 5** Time course of $PGF_{2\alpha}$ (solid columns), $TXB_2$ (light stippled columns) and $PGD_2$ (dark stippled columns) release in response to IL-1$\beta$. Confluent human airway smooth muscle (ASM) cells in 12-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with IL-1$\beta$ 1.0 ng ml$^{-1}$ for the times indicated. $PGF_{2\alpha}$ and $TXB_2$ were determined by EIA and $PGD_2$ was measured by RIA. Results are mean$\pm$s.e.mean for a representative experiment ($n=4$).

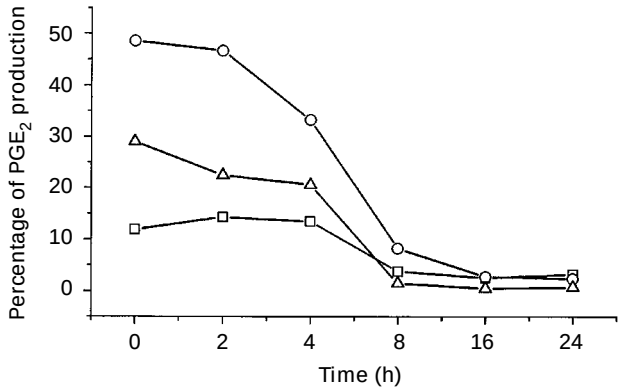


**Figure 6** Relative concentrations of $PGF_{2\alpha}$ ($\square$), $TXB_2$($\bigcirc$) and $PGD_2$ ($\triangle$) compared to $PGE_2$ production at different times after stimulation by IL-1$\beta$ (1.0 ng ml$^{-1}$). Results are the mean of the ratio of $PGF_{2\alpha}$, $TXB_2$ or $PGD_2$ concentration against $PGE_2$ concentration expressed as a percentage of $PGE_2$ production.

### *$PGE_2$ release and COX activity in response to TNF$\alpha$ or IFN$\gamma$*

Treatment of human ASM cells with recombinant human TNF$\alpha$ (6.25–100 ng ml$^{-1}$) or IFN$\gamma$ (1–100 ng ml$^{-1}$) for 24 h caused no substantial release of $PGE_2$ (Figure 7a, $P>0.05$). There was a concentration-related increase of functional COX activity by TNF$\alpha$ treatment ($P<0.01$), but no change was observed after IFN$\gamma$ treatment ($P>0.05$, Figure 7b). Interestingly, Western blot analysis showed that there was no induction in human ASM cells of COX-2 enzyme protein after exposure to either TNF$\alpha$ or IFN$\gamma$ for 24 h (Figure 8, lanes C and D). Reprobing the same blot with anti-COX-1 antibody showed no change in the COX-1 enzyme protein bands (Figure 8, lanes A–D). Cell viability after incubation with TNF$\alpha$ (6.25–100 ng ml$^{-1}$) or IFN$\gamma$ (1.0–100 ng ml$^{-1}$) for 24 h was greater than 95% consistently (MTT assay, data not shown).

### *Effect of various inhibitors on IL-1$\beta$ stimulated $PGE_2$ synthesis and COX activity*

All of the non-steroidal anti-inflammatory drugs (NSAIDs) tested, namely the non-selective COX inhibitors, indomethacin

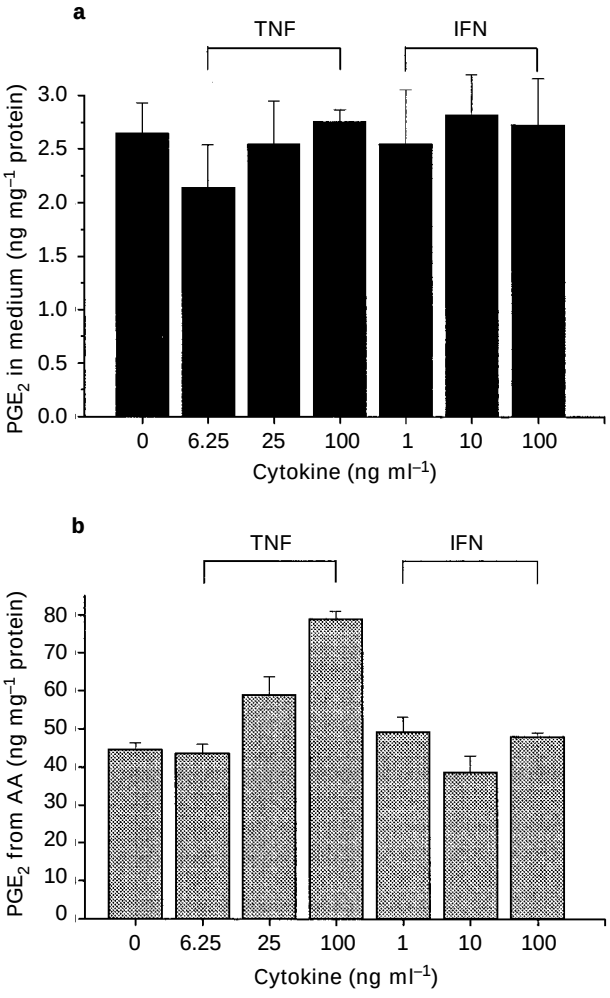


**Figure 7** Effect of tumour necrosis factor-$\alpha$ (TNF$\alpha$) and interferon-$\gamma$ (IFN$\gamma$) on $PGE_2$ release (a) and cyclo-oxygenase (COX) activity (b). Confluent human airway smooth muscle (ASM) cells in 12-well plates were growth arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with various concentrations of TNF$\alpha$ and IFN$\gamma$ for 24 h. $PGE_2$ production was measured by RIA and the COX activity was determined by measuring $PGE_2$ production from exogenous AA (5 $\mu$M, 30 min). Results are mean$\pm$s.e.mean for a representative experiment ($n=4$).

and ibuprofen, and the selective COX-2 inhibitors, NS-398 and nimesulide, completely blocked IL-1$\beta$-induced increase in $PGE_2$ synthesis and COX activity (Figure 9). Cycloheximide (a translation inhibitor), actinomycin D (a transcription inhibitor) and the steroid dexamethasone also suppressed IL-1$\beta$-stimulated $PGE_2$ release and COX activity completely (Figure 9), suggesting that *de novo* synthesis of protein and mRNA was responsible for the IL-1$\beta$-dependent $PGE_2$ synthesis. The effect of protein synthesis inhibitors and dexamethasone on COX-2 and COX-1 induction was further examined by Western blot analysis. As shown in Figure 10, cycloheximide strongly inhibited and actinomycin D and dexamethasone abolished IL-1$\beta$-induced COX-2 induction. However, COX-1 expression was not affected. Cell viability after 24 h treatment by the above reagents and their vehicle (1.0% DMSO) was consistently greater than 95% as measured by MTT assay (data not shown). These results provide further evidence that the induction of COX-2, but not COX-1, is responsible for the IL-1$\beta$-induced prostanoid synthesis and elevation of COX activity in human ASM cells.

## Discussion

We previously found that bovine cultured ASM cells produce $PGE_2$ and that this can be increased by stimulation with arachidonic acid, bradykinin or agents which elevate adenosine 3′:5′-cyclic monophosphate (cyclic-AMP) (Delamere *et al*., 1994; Barry *et al*., 1995). We did not study the spectrum of prostanoids produced by ASM cells, the COX isoenzymes responsible or whether cytokines could induce COX-2. The present series of experiments extend these studies to human ASM and show that human cultured ASM cells also release $PGE_2$ under resting conditions and that this is markedly increased by IL-1$\beta$ stimulation (Figure 1a and c), suggesting that ASM cells may be an important source of $PGE_2$ in human airways. 6-keto-$PGF_{1\alpha}$ was also released under resting and stimulated conditions by these cells (Figure 3) to a similar extent as $PGE_2$. Human ASM cells also produced other prostanoids such as $PGF_{2\alpha}$, $TXB_2$ and $PGD_2$ albeit in much lower concentrations than $PGE_2$ under resting conditions and

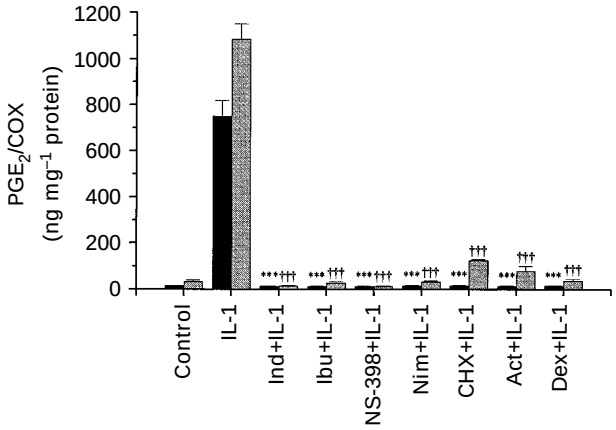


**Figure 9** Effect of various inhibitors on interleukin-1$\beta$ (IL-1$\beta$)-induced $PGE_2$ release (solid columns) and cyclo-oxygenase (COX) activity (stippled columns). Confluent human ASM cells in 12-well plates were growth-arrested for 24 h in serum-free medium. Fresh medium was changed before the experiments and the cells were incubated with 10 $\mu$M indomethacin (Ind), 10 $\mu$M ibuprofen (Ibu), 10 $\mu$M NS-398, 10 $\mu$M nimsulide (Nim), 10 $\mu$M cycloheximide (CHX), 1 $\mu$M actinomycin D (Act) or 10 $\mu$M dexamethasone (Dex), respectively, for 1 h before addition of IL-1$\beta$ (1.0 ng ml$^{-1}$). After 24 h incubation medium was removed for $PGE_2$ assay and the cells were washed with PBS and further incubated with exogenous arachidonic acid (AA, 5 $\mu$M, 30 min), $PGE_2$ generation from AA was determined as COX activity. For the assessment of the effect of NSAIDs on COX activity, after the cells were washed with PBS fresh medium with NSAIDs (identical in concentration for the previous 24 h treatment) was added back to keep the presence of the drugs before the addition of AA. Results are mean$\pm$s.e.mean for a representative experiment ($n=4$). ***, †††Significant difference from IL-1$\beta$ response of $PGE_2$ release and COX activity, respectively, $P<0.001$.

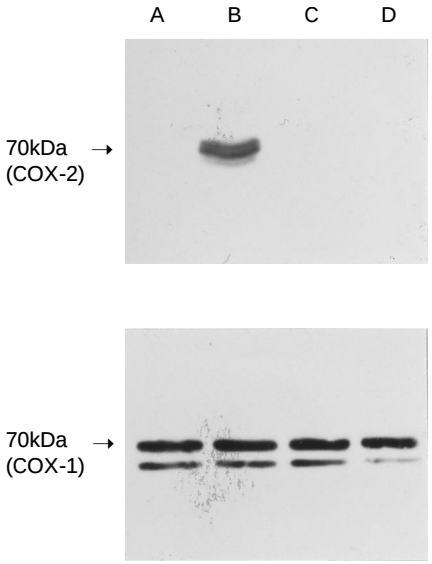


**Figure 8** Effect of tumour necrosis factor-$\alpha$ (TNF$\alpha$) and interferon-$\gamma$ (IFN$\gamma$) on the induction of cyclo-oxygenase-2 (COX-2) in human airway smooth muscle (ASM) cells. Western blot was performed by using specific antibodies to COX-2 and COX-1, respectively. Each lane was loaded with 30 $\mu$g of protein. Lane A: control cells cultured for 24 h, lanes B–D: 1.0 ng ml$^{-1}$ IL-1$\beta$, 100 ng ml$^{-1}$ TNF$\alpha$ and 100 ng ml$^{-1}$ IFN$\gamma$ for 24 h, respectively. The positions and molecular weights of COX-1 and COX-2 were validated by reference to Rainbow coloured molecular weight markers. These blots are representative of similar results obtained at least three times.

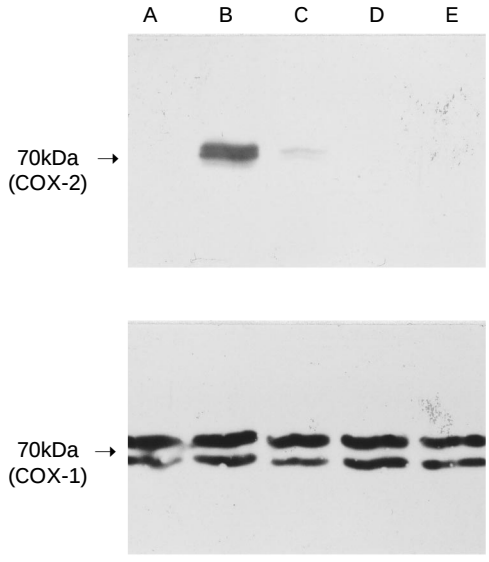


**Figure 10** Effect of protein synthesis inhibitors and dexamethasone on cyclo-oxygenase-2 (COX-2) induction produced by interleukin-1$\beta$ (IL-1$\beta$) in human airway smooth muscle (ASM) cells. Western blot was performed by using specific antibodies to COX-2 and COX-1, respectively. Each lane was loaded with 30 $\mu$g of protein. Lane A: control cells cultured for 24 h, lane B: 1.0 ng ml$^{-1}$ IL-1$\beta$ for 24 h, lanes C–E: same as lane B but pretreated for 1 h with 10 $\mu$M cycloheximide (C), 1 $\mu$M actinomycin D (D) and 10 $\mu$M dexamethasone (E), respectively. The positions and molecular weights of COX-1 and COX-2 were validated by reference to Rainbow coloured molecular weight markers. These blots are representative of similar results obtained at least three times.

these were also increased by IL-1$\beta$ in a concentration- and time-dependent manner (Figures 4 and 5). $PGE_2$ and 6-keto-$PGF_{1\alpha}$ were by far the predominant COX metabolites. This is the first study to look at the spectrum of prostanoids produced by human cultured ASM. It is interesting to note that the ratio of the three low concentration prostanoids ($PGF_{2\alpha}$, $TXB_2$ and $PGD_2$) as compared with $PGE_2$ declined with the time of IL-1$\beta$ stimulation (Figure 6). This suggests that a step beyond COX might also be activated gradually during the stimulation so that relatively more products from COX are converted to $PGE_2$ instead of other metabolites, although these metabolites are also increased. However, the ratio of 6-keto-$PGF_{1\alpha}$ and $PGE_2$ did not seem to have an obvious pattern (data not shown).

Under resting conditions COX-1 was the isoenzyme responsible for prostanoid production in our primary cultures of human tracheal smooth muscle cells. COX-1 was also found to be responsible for $PGE_2$ production under resting conditions in a human cultured bronchial smooth muscle cell line in contrast to three epithelial cell lines where COX-2 was the constitutive and dominant isoform expressed (Asano *et al*., 1996). COX-2 isoenzyme induction by PMA in a cultured human bronchial smooth muscle cell line and COX-2 mRNA expression by IL-1$\beta$ and TNF$\alpha$ in rat tracheobronchial smooth muscle cells have also been observed recently (Asano *et al*., 1996; Vadas *et al*., 1996). However, COX isoenzyme induction by proinflammatory cytokines in human ASM cells and its relation to $PGE_2$ generation and COX activity have not been previously investigated. In our present study, $PGE_2$ release after IL-1$\beta$ treatment was accompanied by a corresponding increase in COX activity (Figure 1b and d, Figure 9) and COX-2 enzyme induction (Figure 2) in a concentration- and time-dependent manner. In contrast, the expression of COX-1 enzyme was not affected by IL-1$\beta$ (Figure 2). The $PGE_2$ synthesis and COX activity were completely inhibited by the non-selective COX enzyme inhibitors, indomethacin and ibuprofen, and selective COX-2 inhibitors, NS-398 and nimsulide (Figure 9). The IL-1$\beta$-induced $PGE_2$ release, COX activity and COX-2 (but not COX-1) induction were also markedly inhibited by cycloheximide and abolished by actinomycin D and dexamethasone (Figures 9 and 10). The results indicate that the IL-1$\beta$-induced increases in $PGE_2$ release and COX activity are attributable mostly to the induction of COX-2 rather than COX-1, which is consistent with the hypothesis that COX-1 is a constitutive isoform whereas COX-2 is an inducible isoform responsible for the generation of prostanoids under inflammatory conditions (Vane *et al*., 1994).

It is not clear whether the consequences of COX-2 induction and prostaglandin production by IL-1$\beta$ in human ASM would be deleterious or beneficial. $PGE_2$ is an important anti-inflammatory mediator and has considerable bronchoprotective effects in the airways (Pavord & Tattersfield, 1995). It is possible therefore that the massive $PGE_2$ production as a result of COX-2 induction is part of a negative feedback mechanism which is exerting a braking effect on the inflammatory response. As $PGI_2$, like $PGE_2$, is also coupled to cyclic AMP elevation, it could have a similar protective effect (Knox & Tattersfield, 1994), but this has not been extensively studied. However, $PGE_2$ at higher concentrations also causes ASM contraction (Sweatman & Collier, 1968; Gardiner, 1975; Armour *et al*., 1989) due to weak agonism at the thromboxane receptor (Knox & Tattersfield, 1995), and $PGF_{2\alpha}$, $TXA_2$ and $PGD_2$ are potent pro-inflammatory modulators which cause bronchoconstriction via the activation of the thromboxane prostanoid receptor (Iwamoto *et al*., 1995; Johnston *et al*., 1995; Aizawa *et al*., 1996). The pro-inflammatory and bronchoconstrictive effects of $PGF_{2\alpha}$, $TXA_2$ and $PGD_2$ and the high concentration of $PGE_2$ released by stimulated ASM cells and other airway cells may eventually outweigh any beneficial effects on $PGE_2$ in ASM cells. The overall effect of IL-1$\beta$-induced prostanoid production in airways *in vivo* is likely to be more complex due to the interaction of several cell types each producing a different spectrum of prostanoids.

TNF$\alpha$ and IFN$\gamma$, like IL-1$\beta$, are both pro-inflammatory cytokines and TNF$\alpha$ has similar effects to IL-1$\beta$ in many cells. Bronchoalveolar lavage fluid from subjects with symptomatic asthma contains significantly elevated levels of a number of pro-inflammatory cytokines including IL-1$\beta$ and TNF$\alpha$ (Mattoli *et al*., 1991; Broide *et al*., 1992), and TNF$\alpha$ and IFN$\gamma$, when used together with IL-1$\beta$ cause increases in $PGE_2$ release, COX activity and induction of COX-2 enzyme (Mitchell *et al*., 1994) and inducible nitric oxide synthase (Robbins *et al*., 1994a) in human lung epithelial cells. We tested the effects of these two cytokines individually on ASM in our study, and found that both failed to cause a significant increase in $PGE_2$ release (Figure 7a) and COX-2 induction (Figure 8). However, TNF$\alpha$, but not IFN$\gamma$, did enhance COX activity in a concentration-dependent manner (Figure 7b). This is consistent with data obtained by Mitchell and co-worker's from human pulmonary epithelial cells (Mitchell *et al*., 1994).

In our study we found that four different NSAIDs all inhibited IL-1$\beta$ induced prostaglandin production. Among them indomethacin and ibuprofen are non-selective COX inhibitors and appear to be more potent in their inhibition of human COX-1 than human COX-2 (Gierse *et al*., 1995), whereas NS-398 and nimesulide are selective COX-2 inhibitors. It has been shown that NS-398 at 10 $\mu$M has no effect on human COX-1 but a strong inhibitory effect on human COX-2 (Gierse *et al*., 1995), and nimesulide at concentrations up to 100 $\mu$M does not inhibit COX-1 (ram seminal vesicles) but does inhibit COX-2 (sheep placenta) (Tavares *et al*., 1995). By using cultured human bronchial smooth muscle cells, Asano found that NS-398 10 $\mu$M had no effect on $PGE_2$ release in response to exogenous AA by these cells which expressed COX-1 (not COX-2) constitutively. However, at the same concentration NS-398 almost completely inhibited the same response by A549 cells (an alveolar type II epithelium-like cell line) which expressed COX-2 (not COX-1) constitutively (Asano *et al*., 1996). In our present study, both NS-398 and nimesulide completely inhibited IL-1$\beta$-induced $PGE_2$ release and COX activity, which led us to speculate that IL-1$\beta$-induced increase in $PGE_2$ release and COX activity was largely attributable to the induction of COX-2 isoenzyme.

The potential therapeutic effects of inhibiting prostanoid release in the airways by NSAIDs for the treatment of asthma are complicated. It is believed that aspirin and other NSAIDs elicit dyspnea in aspirin-sensitive asthmatics by blocking the COX enzyme. However, it is unclear whether this bronchoconstriction is due to the removal of COX product(s) which prevent(s) bronchospasm or to the resultant shunting of AA to the lipoxygenase pathway which generates potent bronchoconstrictor leukotrienes. It has been shown that inhaled $PGE_2$ prevents aspirin-induced bronchoconstriction in aspirin-sensitive asthma (Sestini *et al*., 1996) and 5-lipoxygenase products play a pivotal role in the reaction of aspirin-sensitive asthmatics to aspirin (Israel *et al*., 1993). However, not all asthma patients develop aspirin sensitivity and other factors may be playing a role. NSAIDs may still have some beneficial effects in other types of asthma. For instance, inhaled aspirin and indomethacin prevent the bronchoconstrictive response to inhaled sodium metabisulphite and adenosine monophosphate in asthmatic subjects, probably via the inhibition of local prostanoid synthesis in the airway (Wang *et al*., 1996).

In this study we found that the $PGE_2$ release, COX activity and the induction of COX-2 were all completely inhibited by dexamethasone. Dexamethasone is known to inhibit the production of a number of pro-inflammatory cytokines and the induction of other enzymes (e.g. inducible nitric oxide synthase and phospholipase $A_2$) which generate inflammatory mediators. The inhibitory effect of dexamethasone on COX-2 induction in human ASM cells in our study is similar to that obtained in lung fibroblasts (Endo *et al*., 1995) and pulmonary epithelial cells (Mitchell *et al*., 1994). Dexamethasone also inhibits the induction of nitric oxide synthase in human lung epithelial cells (Robbins *et al*., 1994a) and murine lung epithelial cells (Robbins *et al*., 1994b). Therefore, the therapeutic

benefits of dexamethasone in inflammatory airway diseases such as asthma may be at least partly explained by its inhibition of the expression of inflammatory genes such as COX-2. Clinically, treatment with inhaled corticosteroids results in improvement of symptoms and lung function in subjects with asthma (Vathenen *et al*., 1991).

In conclusion, our studies have shown clearly that $PGE_2$ and 6-keto-$PGE_{1\alpha}$ are the dominant arachidonic acid metabolites produced by human ASM cells and that COX-1 is the sole isoenzyme responsible for $PGE_2$ production under resting conditions. IL-1$\beta$ causes a marked stimulation of prostanoid release and COX activity and this response is mostly mediated by the induction of COX-2 rather than COX-1. Prostanoid release and COX-activity are strongly inhibited by NSAIDs, protein synthesis inhibitors and dexamethasone and the induction of COX-2 is also down-regulated by protein synthesis inhibitors and dexamethasone. These studies may increase our understanding of the pathogenesis of airway inflammation in asthma and human cultured ASM cells may be a useful model to study the regulation of these processes.

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