British Journal of Pharmacology (1998) 125, 1102–1108
http://www.stockton-press.co.uk/bjp

Expression of cyclo-oxygenase-2 in human airway smooth muscle is associated with profound reductions in cell growth

[1,2]Maria G. Belvisi, [2]Michael Saunders, [3]Magdi Yacoub & [4,5]Jane A. Mitchell

[1]Department of Pharmacology, Rhône-Poulenc Rorer, Dagenham Research Centre, Dagenham, Essex RM10 7XS and [2]Department of Thoracic Medicine and [3]Cardiothoracic Surgery, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY; [4]Unit of Critical Care Medicine, Royal Brompton Hospital, Sydney Street, London SW3 6NP

1 It is now accepted that uncontrolled proliferation of human airway smooth muscle (HASM) cells contributes, in many cases, to the chronic stages of asthma. However, the physiological and pathophysiological processes regulating cell growth and division in the airway are not clear. We have recently shown that the immediate early gene, cyclo-oxygenase-2, is induced by cytokines in HASM cells. Since cyclo-oxygenase metabolites, such as prostaglandin (PG) E_2 have been shown to modulate HASM cell growth, we have investigated any autocrine action of endogenously released cyclo-oxygenase-1/2 products on the proliferative responses in these cells.

2 HASM cells were cultured from healthy tissue obtained at lung or heart/lung transplantation. HASM cell proliferation was measured by [^{3}H]-methyl thymidine uptake by cells and by cell counts. Cyclo-oxygenase-2 expression was measured by Western blot analysis and activity measured by the release of PGE_2, by radioimmunoasay.

3 HASM cells proliferated in response to foetal calf serum, a response that was greatly inhibited when cyclo-oxygenase-2 was induced with either interleukin-1β plus tumour necrosis factor-α or interleukin-1β, tumour necrosis factorα plus interferonγ (each at 10 ng ml^{-1}). The inhibitory effect of cytokines on HASM cell proliferation was reversed in a concentration dependent manner by either the mixed cyclo-oxygenase-1/-2 inhibitor, indomethacin or the selective cyclo-oxygenase-2 inhibitor, L-745,337 (each at 10 μM).

4 PGE_2 or the stable analogue of prostacyclin, cicaprost concentration-dependently (0.1 pmol to 1 μM) inhibited serum induced proliferation of HASM cells. By contrast, the TP receptor agonist, U46619 stimulated proliferation of HASM cells when cells were cultured without but not with serum. Other cyclo-oxygenase products, PGD_2, $PGF_{2\alpha}$ had no effect on cellular proliferation at concentrations up to 1 μM.

5 These observations illustrate a profound inhibitory effect of cyclo-oxygenase-2 induction on HASM cell proliferation, possibly via IP or EP receptor activation. Cyclo-oxygenase-2 induction has, thus far, been associated with the pro-inflammatory responses of plasma exudation and oedema formation and is assumed to be an enzyme worthy of selective inhibition in many disease states. However, our observations suggest that cyclo-oxygenase-2 can have an anti-inflammatory, anti-proliferative function in the airways. These observations may have importance in the use and development of therapies for airway disease such as asthma.

Keywords: Prostanoids; airway smooth muscle; cytokines; asthma; proliferation

Introduction

Asthma is a chronic inflammatory disease which is often characterised with thickening of the airway submucosal layer and an increase in airway smooth muscle mass (Dunhill, 1969; Hossain & Heard, 1970). Several groups have examined potential proliferative stimuli using airway smooth muscle cultured from a variety of species including man. Thus, established mitogens for cultured airway smooth muscle include histamine (Panettieri *et al*., 1990), interelukin-1β (De *et al*., 1993), platelet derived growth factor (Hirst *et al*., 1992), thombin (Tomlinson *et al*., 1994) and epidermal derived growth factor (Stewart *et al*., 1994). However, the mechanisms involved in the stimulation of mitogenesis by these agents is unclear. Interestingly many of the agents known to modulate mitogenesis also induce the recently identified isoform of cyclo-oxygenase, cyclo-oxygenase-2 (see Mitchell *et al*., 1995). In fact, cyclo-oxygenase-2 was first identified as a 'mitogen-inducible' immediate early gene (Xie *et al*., 1991). Moreover, arachidonic acid metabolites derived from either cyclo-oxygenase (Tomlinson *et al*., 1994) or lipoxygenase (Noveral & Grunstein, 1992) pathways have potent modulatory actions on airway smooth muscle mitogenesis. Work from our group (Belvisi *et al*., 1997) and that of others (Pang & Knox, 1997) has recently shown that cyclo-oxygenase-2 is expressed in human airway smooth muscle (HASM) cells and that this form is responsible for the production of prostanoids under inflammatory conditions (Belvisi *et al*. 1997). By contrast, the expression of the constitutive cyclo-oxygenase-1 was not altered by cytokines (Belvisi *et al*., 1991). This *in vitro* data has now been consolidated by observations showing that airway biopsies from asthmatic patients express more cyclo-oxygenase-2 than comparable samples from healthy individuals (Sousa *et al*., 1997). Since the autocrine 'functional consequences' of cyclo-oxygenase-2 induction in these cells is not known, we have specifically addressed the effect on proliferation of inducing this enzyme in HASM cells.

[5] Author for correspondence.

Methods

Isolation of HASM cells

As described previously (Hirst *et al*., 1992; Belvisi *et al*., 1997), tracheal rings, from either heart/heart and lung transplantation donors (four female, two male, aged 39–58 years), were dissected under sterile conditions in Hanks buffer saline solution (HBSS; in mM: NaCl 136.8, KCl 5.4, $MgSO_4$ 0.8, Na_2HPO_4. $7H_2O$ 0.4, $CaCl_2$. $2H_2O$ 1.3, $NaHCO_3$ 4.2 and glucose 5.6) supplemented with the antibiotics penicillin (100 U ml^{-1}), streptomysin (100 μg ml^{-1}) and the anti-fungal amphotericin B (2.5 μg ml^{-1}). The smooth muscle layer was dissected free of adherent connective tissue and cartilage, the epithelial layer was removed using a rounded scalpel blade. The smooth muscle section was then incubated for 30 min at 37°C in 5% CO_2/air in HBSS containing 10 mg ml^{-1} bovine serum albumin (BSA) and the enzymes collagenase (type XI, 1 mg ml^{-1}) and elastase (type I, 3.3 U ml^{-1}). After the removal of any remaining connective tissue the smooth muscle was chopped finely and incubated for a further 150 min in the enzyme solution outlined above with the elastase content increased to 15 U ml^{-1}. Cells were centrifuged (100 × g, 5 min) at 4°C to and re-suspended in Dulbeccos modified eagles medium (DMEM) containing heat inactivated foetal calf serum (10% v/v), sodium pyruvate (1 mM), L-glutamate (2 mM), non-essential amino acids (1x) and anti-microbial agents as previously described.

Primary culture of HASM cells

Cells were placed in a tissue culture flask (75 cm^2) with 6 ml of supplemented DMEM and incubated at 37°C in 5% CO_2/air. The cells adhere after approximately 12 h and the culture medium, was replaced after 4–5 days (12 ml) and subsequently every 3–4 days. After approximately 10–14 days the cells reach confluence and are identified by their typical 'hill and valley' appearance and positive immunostaining for α-actin. Cells were passaged and plated onto either 48 well (initial seeding density of 8000 cells/well) or 6 well (initially seeding density of 60,000 cells/well) for thymidine up-take and cell count experiments respectively.

Proliferation assays

At confluence the cells were placed in serum free DMEM (with all other additions present) for 24 h in order to synchronize the cell cycle of the entire population. Medium was then replaced with either serum free DMEM or DMEM containing different concentrations of FCS (2.5, 10 or 20%). In some experiments cytokines (interleukin-1β, IL-1β; tumour necrosis factor-α, TNFα; interferon-γ; IFNγ; 10 ng ml^{-1} for each) or prostanoids (U46619; cicaprost; PGE_2; 0.1 pmol to 1 μM for each), with or without the cyclo-oxygenase-1 and cyclo-oxygenase-2 inhibitor, indomethacin or the cyclo-oxygenase-2 inhibitor, L745337 (10 μM; Chan *et al*., 1995) were also added before serum stimulation. In some experiments 1 m Ci/ml [^{3}H]-methyl thymidine was added, for 2 h, to cells at different times after serum treatment (every 4 h for 48 h). The S-phase of proliferation, defined as the period in which the rate of thymidine taken up into the DNA of cells increased in a linear fashion, began at approximately 20 h. In most experiments [^{3}H]-methyl thymidine was added at 26 h, for 2 h, (established S-phase; Figure 1) after serum/drug treatment. Cells were washed twice with 0.5 ml HBSS to remove any loosely attached radioactive tracer before the addition of 0.5 ml of 5% trichloroacetic acid for 30 min. After two 0.5 ml washes with 95% ethanol, the remaining material on the plate, which represents the DNA fraction, was solubilised by a 30 min incubation with 0.5 ml of 2% Na_2CO_3 in 0.1 M NaOH. Samples were mixed with scintillation fluid (1 : 9 v/v) and radioactivity determined with beta counter.

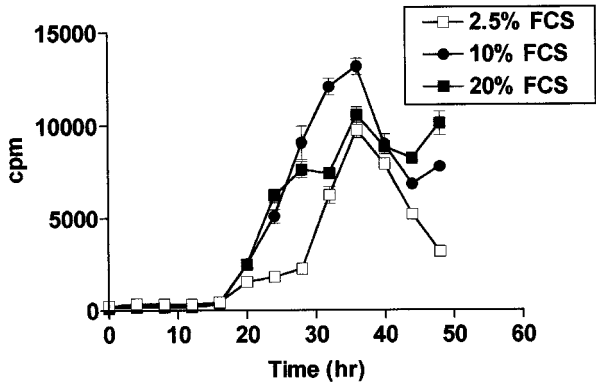

Figure 1 The time course for serum (2.5, 10 and 20% FCS)-induced incorporation of [^{3}H]-methyl thymidine. Cells were 'pulsed' with 1 μCi/ml [^{3}H]-methyl thymidine for 2 h every 4 h up to 48 h. Data are presented as mean ± s.e.mean of four separate experiments.

Cell counts

Cells were treated using protocols outlined in the relevant results section for 48 h. After which cells were removed by the addition of 0.1% trypsin/0.05% EDTA in modified HBSS without calcium and magnesium and cell counts were performed using a Neubauer haemocytometer.

Measurement of PGE_2 by radioimmunoassay

The cyclo-oxygenase metabolite PGE_2 was measured by radioimmunoassay (Mitchell *et al*., 1994; Belvisi *et al*., 1997). Antibodies to PGE_2 had less than 5% cross-reactivity with all other prostaglandins measured (TXB_2, 6-keto $PGF_{1\alpha}$) (Akarseernont *et al*., 1995). Cells were grown in 48 well plates and treated with either cytokines alone or in various combinations in either serum free conditions or in the presence of 10% FCS. In some experiments indomethacin or L-745,337 (10 μM) was added 30 min prior to the cytokine treatments. All treatment were for 28 h when the medium was removed for radioimmunoassay.

Western blot analysis

Western blot analysis was performed as outlined previously (Belvisi *et al*., 1997). Cells were grown to confluence on six well plates and were treated as outlined in the results section. After 28 h the medium was removed and the cells washed with HBSS. The cells were then incubated with an extraction buffer (Tris, 50 mM; EDTA, 10 mM; triton X-100; 1% v/v; phenylmethylsulfonyl fluoride, 1 mM; pepstatin A, 50 μM and leupeptin, 0.2 mM). The resulting cell extract was boiled (10 min) in a ratio of 1 : 1 with gel loading buffer (Tris, 50 mM; SDS, 10% w/v; glycerol, 10% v/v; 2-mercaptoethanol, 10% v/v and bromophenol blue 2 mg ml^{-1}). Samples were centrifuged at 10,000 × *g* for 2 min before being loaded (approximately 10 μg of protein was loaded as determined by a

Bradford protein assay) onto a 4% SDS stacking/7.5% SDS separating gel and subjected to electrophoresis (1.5 h at 125V – 200V). The separated proteins were then transferred to nitrocellulose (BIORAD 1 h at 0.3A). Transfered proteins were blocked (dried minimal-fat milk 25 g and Tween 1.25 ml in PBS solutions 500 ml) for 1 h before being primed for 1 h with a rabbit polyclonal antibody raised against purified sheep placental COX-2 (a gift from Dr Ian Rodger, Merck Frosst, Pointe Claire, Quebec, Canada). The blots were then incubated with a secondary antibody raised to rabbit serum in donkey and conjugated to horseradish peroxidase. Antibody bound protein was visualized by enhanced chemiluminescence (ECL: Amersham plc, U.K.). There was no detectable cross-reactivity of the COX-2 antibody with COX-1 (Belvisi *et al*., 1997).

Cell viability

At the end of each treatment cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan as previously described (Mitchell *et al*., 1993).

Materials

Radiochemicals and ECL reagent were obtained from Amersham International (Amersham, Bucks, U.K.). IL-1β and IFNγ were purchased from Boehringer Manheim (Lewes, East Sussex, U.K.) and TNFα from R&D Systems Europe Ltd. (Abingdon, Oxfordshire, U.K.). The COX-1 and COX-2 standards were purchased from Alexis Corporation Ltd. (Nottingham, U.K.). Amphotericin B, non essential amino acids and sodium pyruvate were purchased from Life Technologies Ltd. (Paisley, U.K.). All other materials were purchased from Sigma Chemical Company (Poole, U.K.).

Statistical analysis

Results are shown as the mean $\pm$ s.e.mean n experiments, cells from at least three separate patients were used for each protocol. Where appropriate data was analysed by Kruskal-Wallis Non-parametric ANOVA test followed by Dunn's test for multiple comparison or, in the case of 'normalized' data by one-sample t-test. All treatments were compared to control values and $P<0.05$ was considered to be significant.

Results

Characterization of FCS induced proliferation of HASM cells

In the absence of any added FCS, very low levels of [^{3}H]-methyl thymidine were incorporated into cells. However, in the presence of either 2.5, 10 or 20% FCS, there was a time dependent incorporation of [^{3}H]-methyl thymidine (Figure 1). The S-phase of proliferation appeared to begin at 20 h and continued to 36 h after serum stimulation. Of the concentrations of FCS used, 10% was optimum at most time points measured. Thus, in all subsequent experiments with [^{3}H]-methyl thymidine, 10% FCS was used and the time point between 26 and 28 h studied. In separate experiments cell counts demonstrated that 10% FCS elicited a 2 fold increase in cell number at 48 h when compared with serum free controls

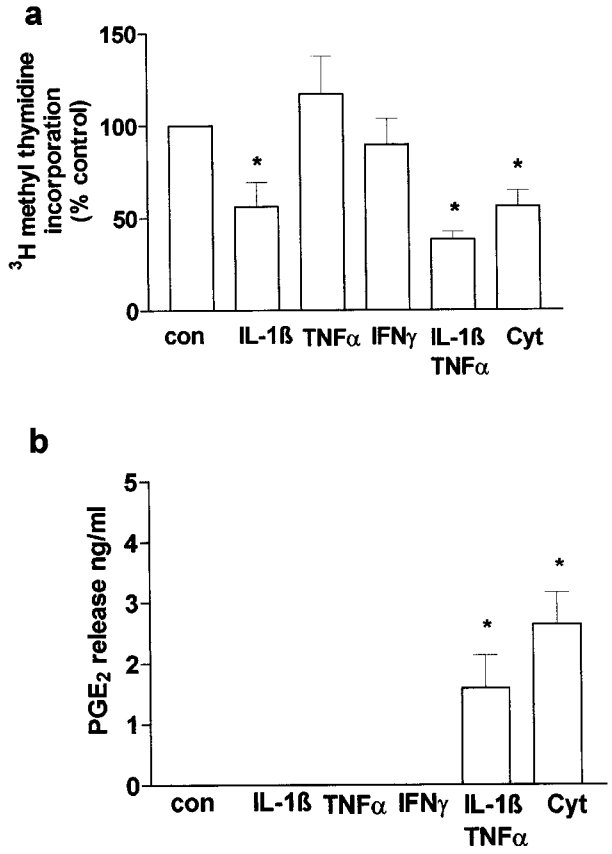

Figure 2 The effect of the pro-inflammatory cytokines IL-1β, TNFα or INFγ or a mixture of all three cytokines (Cyt; 10 ng ml for each) on [^{3}H]-methyl thymidine incorporation (a) and PGE_2 release (b) from HASM cells exposed to serum free conditions for 28 hr. [^{3}H]-methyl thymidine was added 26 h after cytokine stimulation, for 2 h. The data is represented as the mean $\pm$ s.e.mean of six experiments. The level of [^{3}H]-methyl thimidine incorporated under control conditions (i.e. in the absence of serum or added drugs) was 850 ± 120 c.p.m. well^{-1}. *Represents significant differences ($P<0.05$) to the control response calculated by the one-sample t-test for normalized data.

(control $1.12 \pm 0.02 \times 10^6$ cells well^{-1}; 10% FCS, $2.36 \pm 0.16 \times 10^6$ cells well^{-1}; $n=6$).

Effect of cytokines on HASM cell proliferation in the presence and absence of serum: relationship with COX-2 expression and PGE_2 release

The addition of IL-1β (10 ng ml^{-1}), IL-1β plus TNFα (both 10 ng ml^{-1}) or a mixture of IL-1β TNFα and IFNγ (10 ng ml^{-1} of each), to cells stimulated without (Figure 2a) or with 10% FCS (Figure 3a) inhibited [^{3}H]-methyl thymidine incorporation into HASM cells. By contrast, TNFα or INFγ alone did not affect proliferation of these cells (Figures 2a and 3a). In parallel experiments, cells stimulated with cytokines, in the absence of serum, released PGE_2 after IL-1β plus TNFα or with the mixture of IL-1β, TNFα and INFγ but not after IL-1β, TNFα or INFγ alone (Figure 2b). Similar, but more profound, results were obtained when cells were stimulated with cytokines in the presence of serum (Figure 3b). HASM cells expressed very low levels of COX-2 protein (Figure 4) when cultured in 10% FCS. The expression of COX-2 protein was greatly increased when cells were stimulated with IL-1β alone or in combination with

TNFα or when cells were stimulated with the cytokine mixture (Figure 4).

In separate experiments, IL-1β + TNFα and the cytokine mixture inhibited the increase in cell number stimulated with 10% FCS by 42 ± 4.5% and 54 ± 5.7% ($n = 4$; $P < 0.05$ by one sample t-test for normalized data) respectively. By contrast, IL-1β, TNFα or IFNγ alone had no effect on cell number ($n = 4$).

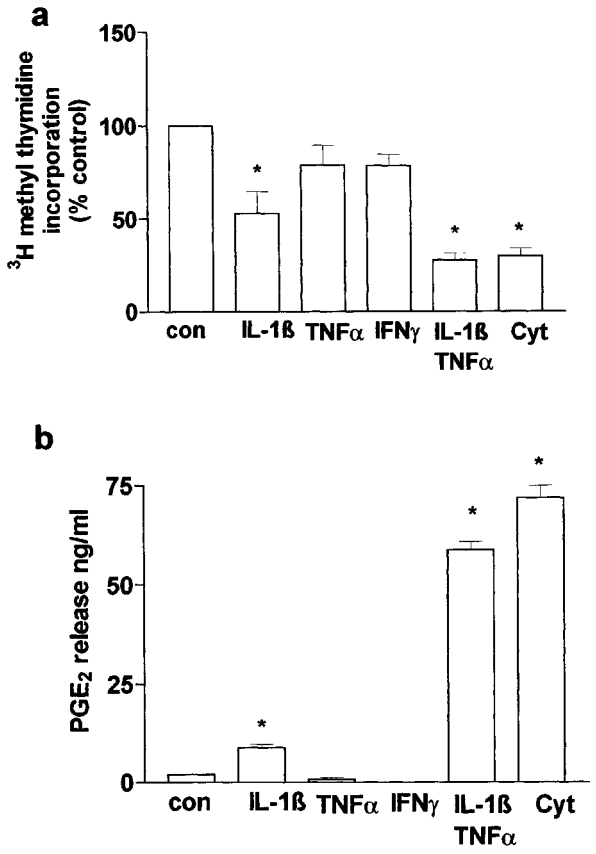

Figure 3 The effect of the pro-inflammatory cytokines IL-1β, TNFα or INFγ or a mixture of all three cytokines (Cyt; 10 ng/ml for each) on [^{3}H]-methyl thymidine incorporation (a) and PGE_2 release (b) from HASM cells exposed to 10% FCS for 28 h. [^{3}H]-methyl thymidine was added 26 h after serum/cytokine stimulation, for 2 h. The data is represented as the mean ± s.e of six experiments. The level of [^{3}H]-methyl thimidine incorporated under control conditions (ie in the presence of serum but in the absence of added drugs) was 12000 ± 1500 c.p.m. well^{-1}. *Represents significant differences ($P < 0.05$) to the control response calculated by the one-sample t-test for normalized data.

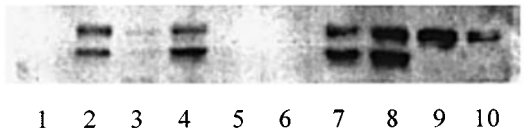

Figure 4 Expression of COX-2 protein in HASM cells showing a representative Western blot (n = 3) using a specific antibody to COX-2 which recognises a band of approximately 70 kDa. Each lane was loaded with 10 μg of protein for samples and 10 ng of protein for the COX standards. Lane 1, COX-1 standard; Lane 2, COX-2 standard; Lane 3, extracts from cell exposed to 10% FCS alone; Lanes 4-10, extracts from HASM cells exposed to 10% FCS + Lane 4, IL-1β (10 ng ml); Lane 5, TNFα, (10 ng ml^{-1}); Lane 6, IFNγ (10 ng ml^{-1}); Lane 7, IL-1β + TNFα, (both 10 ng ml^{-1}); Lane 8, IL-1β + TNFα + IFNγ (all 10 ng ml^{-1}); Lane 9, pre-treated with indomethacin (10 μM; for 30 min) prior to treatment with IL-1β + TNFα + IFNγ (all 10 ng ml^{-1}); Lane 10, pre-treated with L-745,337 (10 μM; for 30 min) prior to treatment with IL-1β + TNFα + IFNγ (all 10 ng ml^{-1}). All treatments were for 28 h. The blot shown is representative of three separate experiments.

Role of COX-1 and COX-2 in the proliferation of HASM cells

The COX-1/COX-2 inhibitor indomethacin (Figure 5b; 10 μM) and the COX-2 selective inhibitor L-745,337 (Figure 6b; 10 μM) inhibited PGE_2 release generated by HASM cells stimulated with serum. In addition, both indomethacin and L-745,337 significantly increased [^{3}H]-methyl thymidine incorporation by these cells (Figures 5a and 6a). Pre-incubation (30 min prior to cytokines) of HASM cells with either indomethacin or L-745,337 (both 0.01 – 10 μM) concentration-dependently inhibited the release of PGE_2 and reversed the decrease in [^{3}H]-methyl thymidine uptake stimulated by the mixture of IL-1β, TNFα and INFγ (Figures 5a and 6a). In addition, the reduction in cell numbers caused by the mixture of IL-1β, TNFα and IFNγ was reversed by either indomethacin (10 μM) or L-745,337 (10 μM), ($n = 4$; data not shown).

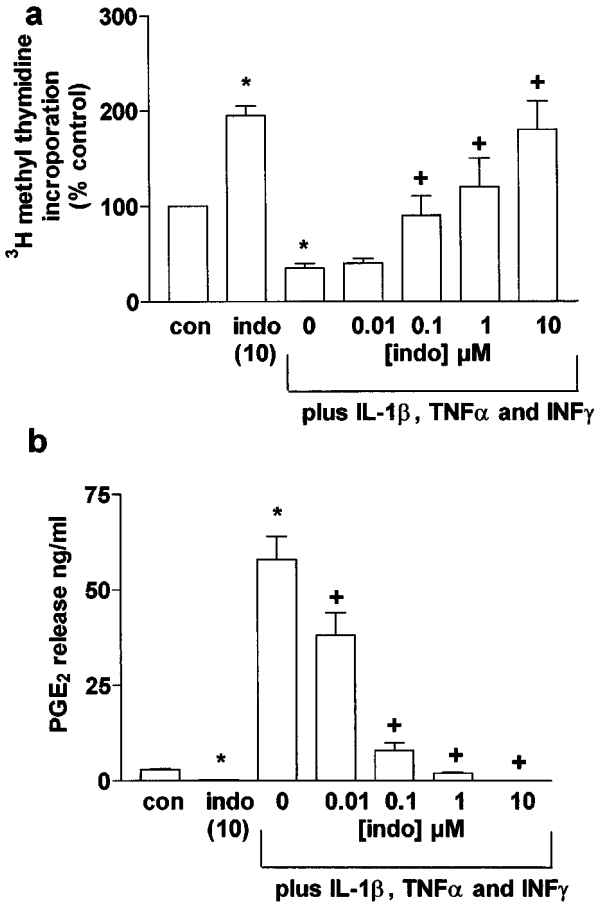

Figure 5 Effect of the COX-1/COX-2 inhibitor indomethacin (0.01-10 μM) on 10% FCS induced and cytokine mediated inhibition of [^{3}H]-methyl thymidine incorporation (a) and PGE_2 release (b). [^{3}H]-methyl thymidine was added 26 h after serum/cytokine and NSAID addition, for 2 h. The level of [^{3}H]-methyl thimidine incorporated under control conditions (i.e. in the presence of serum but in the absence of added drugs) was 9500 ± 1200 c.p.m. well^{-1}. The results are presented as the mean ± s.e.mean from six experiments.*Represents significant differences ($P < 0.05$) to the control response calculated by the one-sample t-test for normalized data. + Represents significant differences between responses of cells treated with cytokines alone or in the presence of different concentrations of indomethacin.

Effect of exogenous prostanoids proliferation of HASM cell

In cells pre-treated with indomethacin (10 μM) to block endogenous prostanoid production, PGE_2 and cicaprost caused a concentration-dependent inhibition of both 'basal' and 10% FCS-induced proliferation of HASM cells. By contrast, PGD_2, $PGF_{2\alpha}$ had no significant effect, at concentrations up to 1 μM, on [^{3}H]-methyl thymidine uptake by HASM cells, stimulated with or without serum ($n=9$; data not shown). U46619 (TP agonist) stimulated [^{3}H]-methyl thymidine uptake in a concentration dependent manner in cells prepared without serum, but not in cells stimulated with serum (Figure 7).

Discussion

Here we show that FCS stimulates HASM cells to proliferate and to express cyclo-oxygenase-2. Moreover, the level of cyclo-oxygenase-2 protein and activity was increased greatly when cells were stimulated with IL-1β together with TNFα. These observations are in keeping with those recently reported by our group (Belvisi *et al*., 1997) and others (Pang & Knox, 1997). In parallel with increased cyclo-oxygenase-2 expression, we observed a reduction in the proliferative response of HASM cells treated with cytokines.

The finding that serum stimulates cyclo-oxygenase-2 expression in HASM cells is in keeping with observations made in a range of other cell types (see Mitchell *et al*., 1995). The active components of serum responsible for stimulating cyclo-oxygenase-2 induction in our study and others are most likely to be growth factors such as platelet derived growth

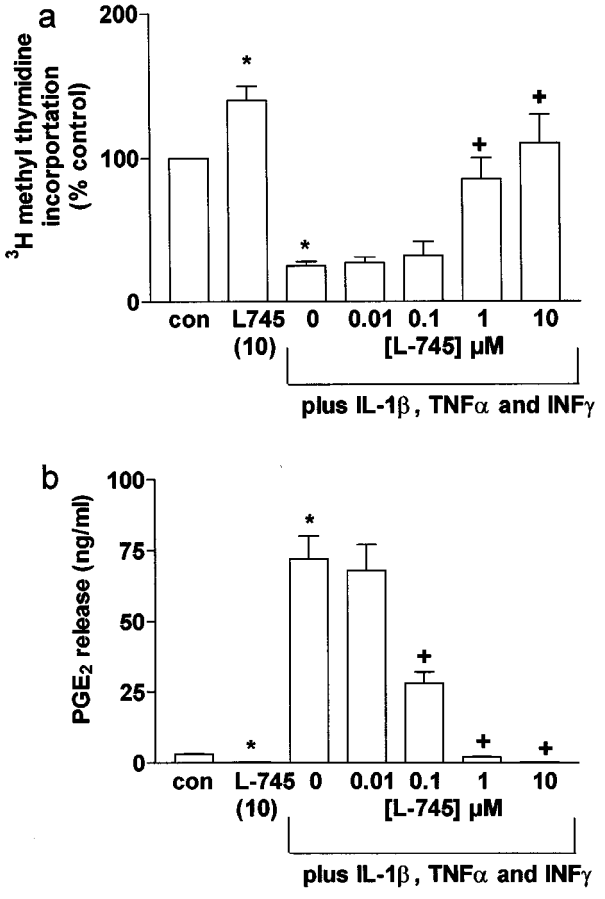

Figure 6 Effect of the COX-2 selective inhibitor L-745,337 (0.01-10 μM) on 10% FCS-induced and cytokine mediated inhibition of [^{3}H]-methyl thymidine incorporation (a) and PGE_2 release (b). [^{3}H]-methyl thymidine was added 26 h after serum/cytokine and NSAID addition, for 2 h. The results are presented as the mean$\pm$s.e.mean from six experiments. The level of [^{3}H]-methyl thimidine incorporated under control conditions (ie in the presence of serum but in the absence of added drugs) was 9500$\pm$1200 c.p.m. well^{-1}. *Represents significant differences ($P<0.05$) to the control response calculated by the one-sample *t*-test for normalized data. +represents significant differences between responses of cells treated with cytokines alone or in the presence of different concentrations of L-745,337.

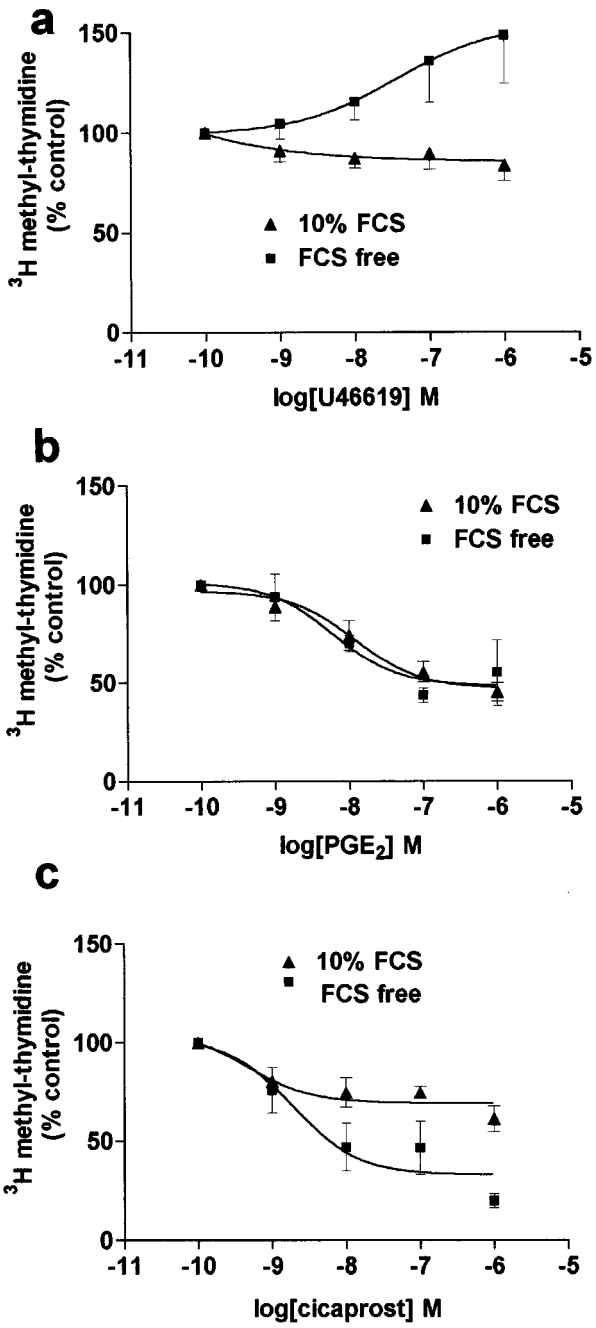

Figure 7 This figure demonstrates the effect of exogenously added prostanoids on [^{3}H]-methyl thymidine incorporation in HASM cells pre-treated with 10 μM indomethacin to remove any endogenous prostanoid generation before exposure to serum free (control [^{3}H]-methyl thymidine levels, 720$\pm$150 c.p.m. well^{-1}) or 10% FCS (control [^{3}H]-methyl thymidine levels, 9850$\pm$1900 CPM well^{-1}). [^{3}H]-methyl thymidine was added 26 h after all additions, for 2 h. The results are presented as the mean$\pm$s.e.mean of nine experiments.

factor which readily induce this enzyme in cultured cells. In addition we found that cyclo-oxygenase-2 expression after serum stimulation limited the proliferative response. This was illustrated by findings that either indomethacin (mixed cyclo-oxygenase-1 and cyclo-oxygenase-2 inhibitor) or L-745,337 (selective cyclo-oxygenase-2 inhibitor; Chan *et al*., 1995) significantly increased serum-induced proliferation of HASM cells. These observations are in contrast to others using intestinal epithelial cells (Shang *et al*., 1997) or colon cancer cells, where the expression of cyclo-oxygenase-2 by mitogenic stimuli contributed to cell growth. The reasons for differences observed here with HASM cells and in studies using intestinal epithelial cells are not known but may be related to differences in the profile of cyclo-oxygenase-2 products produced by and the expression of prostanoid receptors on the different cell types, as discussed below.

When cells were treated with IL-1β, either alone or in combination with TNFα and INFγ, cyclo-oxygenase-2 protein and activity was increased and proliferation was reduced. Moreover, this inhibition was completely reversed when cyclo-oxygenase-2 was blocked. Interestingly, we also observed that both indomethacin and L-745337 greatly reduced the presence of a 60kd breakdown product of COX-2 (Chan *et al*., 1995). We have recently made similar observations in human arterial and venous smooth muscle cells, where the effects of L-745337 on the 60 kd band were reversed by exogenous PGE_2 (Bishop-Bailey *et al*., 1998). Thus, prostanoid products of COX-2 may well participate in the breakdown of the protein.

Observations made in this study may have important implications for the pathology of asthma. In asthma, airway cells are likely to be exposed to the same inflammatory cytokines that induce cyclo-oxygenase-2, either continuously or during periods when the disease is severe. Evidence for this has recently been published by Sousa *et al*. (1997), who showed that cyclo-oxygenase-2 expression was increased 6 fold in the airways of asthmatic patients. Clearly if our observations *in vitro* are representative of events *in vivo*, cyclo-oxygenase-2 expression represents an important anti-proliferative enzyme in human asthma. Our observations with IL-1β are in contrast to others showing that IL-1β stimulates proliferation of guinea pig cultured airway smooth muscle cells (De *et al*., 1993). However, De and co-workers showed that indomethacin had to be present in the culture medium for IL-1β to stimulate proliferation. Thus, in guinea-pig cells, like human cells, the induction of COX-2 is likely to be an anti-proliferative response. We found that, in addition to PGE_2, the prostacyclin analogue cicaprost also inhibited proliferation of HASM cells. Together these observations suggest that activation of adenylyl cyclase and formation of cyclic AMP, *via* EP and/or IP receptor stimulation, mediates the anti-proliferative effect of cyclo-oxygenase-2 in our cells. This is supported by studies showing that PGE_2 and cell permeable analogues of cyclic AMP inhibit proliferation in guinea-pig and HASM cells (Florio *et al*., 1994; Tomlinson *et al*., 1995). In support of this, a recent study using human vascular smooth muscle cells showed that PGE_2 inhibits proliferation *via* activation of the cyclic AMP-dependent protein kinase (Bornfeldt *et al*., 1997).

In conclusion, our data shows that cyclo-oxygenase-2 is induced when HASM cells are simulated to proliferate and further enhanced by inflammatory cytokines. In both cases cyclo-oxygenase-2 exerts a powerful anti-proliferative effect on the cells that is, most likely, mediated by a mixture of PGE_2 and prostacyclin. From these studies we suggest that cyclo-oxygenase-2 inhibitors may have side effects in some asthmatic patients who have ongoing airway remodelling modulated by prostanoids.

The authors acknowledge the kind assistance of Dr Stewart Hirst and the helpful discussion of Professor Peter Barnes. Jane A. Mitchell is a recipient of a Wellcome Career Development award. This work was partly funded by the British Lung Foundation.

References

AKARASEREENONT, P., MITCHELL, J.A., THIEMERMANN, C. & VANE, J.R. (1995). Comparison of the induction of cyclo-oxygenase and nitric oxide synthase by endotoxin in endothelial cells and macrophages. *Eur. J. Pharmacol.,* **173,** 121 – 128.

BELVISI, M.G., SAUNDERS, M.A., HADDAD, E.-B., HIRST, S.J., YACOUB, M.H., BARNES, P.J. & MITCHELL, J.A. (1997). Induction of cyclo-oxygenase-2 by cytokines in human cultured airway smooth muscle cells: novel inflammatory role of this cell type. *Br. J. Pharmacol.,* **120,** 910 – 916.

BISHOP-BAILEY, D., PEPPER, J.R., LARKIN, S.L. & MITCHELL, J.A. (1998). Differential induction of Cyclo-oxygenase-2 in arterial and venous smooth muscle cells: role of endogenous prostanoids. *Athero. Throm. & Vasc. Biol.,* in press.

BORNFELDT, K.E., CAMPBELL, J.S., KOYAMA, H., ARGAST, G.M., LESLIE, C.C., RAINES, E.W., KREBS, E.G. & ROSS, R. (1997). The mitogen-activated protein kinase pathway can mediate growth inhibition and proliferation in smooth muscle cells. Dependence on the availability of downstream targets. *J. Clin. Invest.,* **100,** 875 – 885.

CHAN, C.C., BOYCE, S., BRIDEAU, C., FORD-HUTCHINSON, A.W., GORDON, R., GUAY, D., HILL, R-G., LI, C.S., MANCINI, J., PENNETON, M., PRASIT, P., RASORI, R., RIENDEAU, D., ROY, P., TAGARI, P., VICKERS, P., WONG, E. & RODGER, I.W. (1995). Pharmacology of a selective cyclo-oxygenase-2 inhibitor, L-745,337: a novel nonsteriodal anti-inflammatory agent with an ulcerogenic sparing effect in rat and non-human primate stomach. *J. Pharmacol. Exp. Ther.,* **274,** 1531 – 1537.

DE, S., ZELASNY, E.T., SOUTRADA, J.F. & SOUHRADA, M. (1993). Interleukin-1β stimulates the proliferation of cultured airway smooth muscle via platelet derived growth factor. *Am. J. Repir. Cell. Mol. Biol.,* **9,** 645 – 651.

DUNNILL, M.S., MASSARELLE, G.R. & ANDERSON, J.A. (1969). A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chromic bronchitis and in emphysema. *Thorax,* **24,** 175 – 179.

FLORIO, C., MARTIN, J.G., STYHLER, A. & HEISLER, S. (1994). Antiproliferative effect of prostaglandin E_2 in cultured guinea pig smooth muscle cells. *Am. J. Physiol.,* **266,** L131 – L137.

HOSSAIN, S. & HEARD, B.E. (1970). Hyperplasia of bronchial muscle in chronic bronchitis. *J. Pathol.,* **101,** 171 – 184.

HIRST, S.J., BARNES, P.J. & TWORT, C.H.C. (1992). Quantifying proliferation of cultured human and rabbit airway smooth muscle cells in response to serum and platelet derived growth factor. *Am. J. Respir. Cell Molec. Biol.,* **7,** 574 – 581.

MITCHELL, J.A., AKARASEREENONT, P., THIEMERMANN, C., FLOWER, R.J. & VANE, J.R. (1993). Selectivity of non-steroidal anti-inflammatory drugs as inhibitors of constitutive and inducible cyclo-oxygenase. *Proc. Natl. Acad. Sci.,* **90,** 11693 – 11697.

MITCHELL, J.A., BELVISI, M.G., AKARASEREENONT, P., ROBBINS, R.A., KWON, O.J., CROXTALL, J., BARNES, P.J. & VANE, J.R. (1994). Induction of cyclo-oxygenase-2 by cytokines in human pulmonary epithelial cells: regulation by dexamethasone. *Br. J. Pharmacol.,* **113,** 1008 – 1014.

MITCHELL, J.A., WILLIAMS, T.J. & LARKIN, S.L. (1995). Cyclo-oxygenase-2: regulation and relevance in inflammation. *Biochem. Pharmacol.,* **50,** 1535 – 1542.

NOVERAL, J.P. & GRUSTEIN, M.M. (1992). Role and mechanism of thromboxane-induced proliferation of cultured airway smooth muscle cells. *Am. J. Phaysiol.,* **263,** L317 – L324.

PANETTIERI, R.A., YADVISH, P.A., KELLY, A.M., RUBINSTEIN, N.A. & KOTLIKOFF, M.I. (1990). Histamine stimulates proliferation of airway smooth muscle and induces c-fos expression. *Am. J. Physiol.,* **259,** L365–371.

PANG, L. & KNOX, A.J. (1997). Effect if interleukin-1β, tumour necrosis factor and interferon-gamma on the induction of cyclo-oxygenase-2 in cultured human airway smooth muscle cells. *Br. J. Pharmacol.,* **121,** 579–587.

SHANG, G.G., SHAO, J., SHENG, H., HOOTON, E.B., ISAKSON, P.C., MORROW, J.D., COFFEY, R.J., DUBOIS, R.N. & BEAUCHAMP, R.D. (1997). A selective cyclooxygenase 2 inhibitor suppresses the growth of H-ras-transformed rat intestinal epithelial cells. *Gastroenterology,* **113,** 1883–1891.

SOUSA, A.R., PFISTER, R., CHRISTIE, P.E., LANE, S.J., NASSER, S.M., SCHMITZ-SCHUMANN &LEE, T.H. Enhanced expression of cyclo-oxygenase isoenzyme 2 (COX-2) in asthmatic airways and its cellular distribution in aspirin sensitive asthma. *Thorax,* **52,** 940–945.

STEWART, A.G., GRIGORIADIS, G. & HARRIS, T. (1994). Mitogenic actions of endothelin-1 and epidermal growth factor in cultured airway smooth muscle. *Clin. Exp. Pharmacol. Physiol.,* **21,** 277–285.

TOMLINSON, P.R., WILLSON, J.W. & STEWART, A.G. (1994). Inhibition by salbutamol of the proliferation of human airway smooth muscle cells grown in culture. *Br. J. Pharmacol.,* **111,** 641–647.

TOMLINSON, P.R., WILSON, J.W. & STEWART, A.G. (1995). Salbutamol inhibits the proliferation of human airway smooth muscle cells grown in culture: relationship to elevated cAMP levels. *Biochem. Pharmacol.,* **49,** 1809–1819.

XIE, W.L., CHIPMAN, J.G., ROBERTSON, D.L., ERIKSON, R.L. & SIMMONS, D.L. (1991). Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci.,* **88,** 2696–2696.

(Received April 23, 1998
Revised July 2, 1998
Accepted July 7, 1998)