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Interleukin-1α and tumour necrosis factor-α modulate airway smooth muscle DNA synthesis by induction of cyclo-oxygenase-2: inhibition by dexamethasone and fluticasone propionate

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1 Previous studies have established that glucocorticoids inhibit airway smooth muscle DNA synthesis. The effects of a combination of the pro-inflammatory cytokines, interleukin-1α (IL-1α) and tumour necrosis factor-α (TNF-α) on the inhibition of DNA synthesis by glucocorticoids in human cultured airway smooth muscle have now been investigated, since these cytokines are chronically expressed in asthmatic airways.

2 Thrombin (0.3 u ml^{-1}) and basic fibroblast growth factor (bFGF, 300 pM) stimulated increases in DNA synthesis which were concentration-dependently inhibited by dexamethasone (1–1000 nM).

3 The cytokine mixture, comprising IL-1α (0.01 and 0.1 pM) and TNF-α (3 and 30 pM), directly evoked increases in DNA synthesis which were attenuated by dexamethasone. However, the cytokine mixture prevented responses to bFGF or thrombin.

4 Paradoxically, in the presence of the cytokine mixture and bFGF, dexamethasone (1–1000 nM) concentration-dependently increased DNA synthesis. Furthermore, neither dexamethasone (100 nM) nor fluticasone propionate (1 nM) inhibited DNA synthesized in response to bFGF/cytokine mixture combination and dexamethasone was similarly inactive against the thrombin/cytokine mixture.

5 The levels of prostaglandin E_2 (PGE_2), an established inhibitor of airway smooth muscle DNA synthesis, remained below the limits of assay detection (0.05 nM) under basal conditions or following stimulation with either thrombin or bFGF. In contrast, the cytokine mixture alone, and in the presence of thrombin or bFGF, induced biologically active levels of PGE_2. Dexamethasone (100 nM), the non-selective cyclo-oxygenase (COX) inhibitor indomethacin (3 μM) or the selective COX-2 inhibitor L-745,337 (0.3 μM) completely inhibited synthesis of PGE_2.

6 Neither indomethacin (3 μM) nor L-745,337 (0.3 μM) influenced thrombin- or bFGF-induced DNA synthesis. However, each COX inhibitor enhanced DNA synthesis in cytokine-treated cells.

7 In unstimulated airway smooth muscle cells, COX-1, but not COX-2 protein was detectable by Western blotting. The induction of COX-2 protein by the cytokine mixture was attenuated by dexamethasone (100 nM), whereas the level of COX-1 protein was unaffected by either the cytokines or by dexamethasone.

8 Cytokine-induced, COX-2-dependent eicosanoid production inhibits DNA synthesis. The paradoxical increase in DNA synthesis observed in glucocorticoid treated airway smooth muscle stimulated by cytokine/bFGF combinations may be explained by the ability of glucocorticoids to repress COX-2 induction and prevent cytokine-induction of the DNA synthesis inhibitor, PGE_2.

Keywords: Asthma; cytokines; interleukin-1α; tumour necrosis factor-α; dexamethasone; glucocorticoids; human airway smooth muscle

Abbreviations: ASM, airway smooth muscle; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; COX, cyclo-oxygenase; cyt mix, cytokine mixture; dex, dexamethasone; DMSO, dimethyl sulphoxide; DMEM, Dulbecco's Modified Eagle's Medium; FP, fluticasone propionate; FCS, foetal calf serum; IL-1α, interleukin-1α; PECAM-1, platelet endothelial cell adhesion molecule-1; PBS, phosphate buffered saline; PGE_2, prostaglandin E_2; RANTES, regulated upon activation normal T cell expressed and secreted; TNF-α, tumour necrosis factor-α

Introduction

Asthma is a disease characterized by the presence of chronic airways inflammation and airway wall remodelling. Remodelling results in part from smooth muscle hyperplasia (Dunnill, 1969; Heard & Hossain, 1973; Hossain, 1973) and hypertrophy (Ebina *et al*., 1993) and may explain a major part of the airway hyperresponsiveness associated with asthma (James *et al*., 1989; Kuwano *et al*., 1993; Pare *et al*., 1991; Wiggs *et al*., 1992). Studies from this and other laboratories have identified biologically diverse stimuli for proliferation of cultured human airway smooth muscle which include peptide growth factors, proteases, low molecular weight bronchoconstrictors and some pro-inflammatory cytokines (see Stewart *et al*., 1995c for review). The major anti-asthma drug classes, β_2-adrenoceptor agonists and glucocorticoids, attenuate airway smooth muscle proliferation (Schramm *et al*., 1996; Stewart *et al*., 1995a; 1997; Tomlinson *et al*., 1995; Young *et al*., 1995).

Pro-inflammatory cytokines play an important role in perpetuating the airways inflammatory response in asthma and biologically active amounts have been detected in bronchoalveolar lavage fluid taken from asthmatic patients (Broide *et al*., 1992; Cembrzynska-Nowak *et al*., 1993; Mattoli *et al*., 1991; Walker *et al*., 1992). We have previously shown

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that low concentrations (3–30 pM) of TNF-α stimulate and higher concentrations (300 pM) inhibit airway smooth muscle DNA synthesis, both effects being inhibited by dexamethasone (Stewart *et al*., 1995b).

Transbronchial biopsy of asthmatic airways and immunoassay of bronchoalveolar lavage fluids indicate that airway smooth muscle *in vivo* may be chronically exposed to a multitude of pro-inflammatory cytokines including TNF-α (Broide *et al*., 1992; Cembrzynska-Nowak *et al*., 1993; Gosset *et al*., 1991), interleukin-1 (Broide *et al*., 1992; Cembrzynska-Nowak *et al*., 1993; Mattoli *et al*., 1991), interleukin-4 (Walker *et al*., 1992; Ying *et al*., 1995), interleukin-5 (Walker *et al*., 1992; Ying *et al*., 1995), interferon γ (Cembrzynska-Nowak *et al*., 1993), granulocyte-macrophage colony-stimulating factor (Broide *et al*., 1992; Mattoli *et al*., 1991; Woolley *et al*., 1994) and transforming growth factor β_1 (Redington *et al*., 1997). Previous studies have established that some of these cytokines elicit responses in cultured airway smooth muscle comprising cell proliferation and hypertrophy (Amrani *et al*., 1996; De *et al*., 1993; Stewart *et al*., 1995b) and eicosanoid production (Belvisi *et al*., 1997; Pang & Knox, 1997). Human airway smooth muscle itself is a source of cytokines and chemokines including granulocyte-macrophage colony-stimulating factor (Saunders *et al*., 1997), interleukin-8 (John *et al*., 1998) and regulated upon activation normal T cell expressed and secreted (RANTES) (John *et al*., 1997). These latter findings, and evidence of eicosanoid production, have led to the concept that airway smooth muscle may be an important source of inflammatory mediators.

In this study, we have re-evaluated the effects of glucocorticoids on DNA synthesis in airway smooth muscle exposed to a mixture of IL-1α and TNF-α, since the presence of these cytokines has been well documented in airway inflammation and it is therefore relevant to consider how they modify the pharmacological actions of glucocorticoids.

Methods

Cell culture

Human airway smooth muscle (ASM) cell cultures were generated from bronchi (0.5–2 cm diameter) obtained from lung resected from heart-lung transplant recipients. Smooth muscle was microdissected with the aid of a binocular operating microscope and enzymatically digested with collagenase and elastase to generate cell suspensions which were then cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% w/v foetal calf serum (FCS) as previously described (Stewart *et al*., 1997). Cells were passaged weekly at a 1 : 3 split ratio by a 10 min exposure to 0.5% w/v trypsin (in phosphate buffered saline containing 1 mM EDTA) generating a cell density of approximately 1×10^4 cells per cm^2. Cells at passage numbers 4–14 were used for experiments, a period in which there is no relationship between cell passage number and responsiveness to growth factors or inhibitors and the expression of smooth muscle α-actin is maintained (Stewart *et al*., 1997).

Immunohistochemistry

The cellular composition of the cultures was determined using expression of smooth muscle specific α-actin and myosin. Cells were subcultured into 8-well glass chamber slides, allowed to grow to confluency, after which the medium was replaced with serum-free DMEM (containing 0.25% w/v bovine serum albumin; 1% v/v Monomed A - comprising insulin, transferin and selenium) for 7 days to render the cells quiescent and to allow re-expression of contractile proteins (Panettieri *et al*., 1989). Cells were then fixed in ice-cold acetone for 20 s and stored for up to 4 weeks at 4°C before staining. Immunohistochemical staining was performed as described previously (Stewart *et al*., 1997) using anti-smooth muscle α-actin, anti-smooth muscle myosin or anti-platelet endothelial cell adhesion molecule-1 (PECAM-1, mouse monoclonal CD31, a marker of endothelial cells) followed by incubation with a horseradish-peroxidase (HRP) conjugated secondary antibody. Staining of the fixed cells was observed by light microscopy. Each of the cell cultures stained positively for α-actin and myosin in over 95% of the cells, and there was no detectable PECAM-1 expression.

DNA synthesis

Cells were subcultured into 24 well plates at approximately $1.5–2 \times 10^4$ cells per cm^2 and allowed to grow for 72 h to monolayer confluency in DMEM containing 10% FCS. Thereafter, the cells were growth arrested in medium (containing 0.25% bovine serum albumin) without FCS for a further 24 h to synchronize the cells in G_0 of the cell cycle. Cells in allotted wells were then pretreated with the pro-inflammatory cytokine mixture for 24 h before the addition of a maximally effective concentration of thrombin (0.3 u ml^{-1}) or bFGF (300 pM) (Tomlinson *et al*., 1994). Glucocorticoids were added 60 min prior to mitogen addition. The growth supplement Monomed A (1% v/v) was added to all wells at the time of mitogen stimulation to provide progression factors essential for the mitogenic activity of growth factors such as thrombin, epidermal growth factor, and bFGF (Tomlinson *et al*., 1994; Stewart *et al*., 1995a). Twenty four hours after the addition of thrombin or bFGF, the cells were incubated for a further 4 h in 1 μCi ml^{-1} [^{3}H]-thymidine to allow incorporation into newly synthesized DNA and then harvested as described previously (Stewart *et al*., 1997) with a binding harvester (Packard Filtermate 196, Canberra Packard, Melbourne, Australia). Radioactivity was measured by liquid scintillation counting (Packard Topcount, Canberra Packard, Melbourne, Australia).

Radioimmunoassays

Prostaglandin E_2 levels were determined in a volume of 100 μL of the unextracted culture medium harvested immediately prior to the addition of [^{3}H]-thymidine. The radioimmunoassay of PGE_2 was carried out using a commercially available antibody (UBI, Lake Placid, NY, U.S.A.) and methods of Salmon (1978) as described previously (Lim & Stewart, 1991). The limit of detection of the assay was 0.05 nM, and its cross-reactivities (as determined by the supplier) were as follows: PGE_2 100%, PGE_1 100%, $PGF_{2\alpha}$ 3%, $PGF_{1\alpha}$ 2% and TXB_2 0%.

Immunoblot analysis

Human ASM cells were grown in six well plates and stimulated under conditions identical to those used for estimation of DNA synthesis. After incubations with mitogen for 4 h, the cells were washed twice in ice-cold PBS and lysed on ice for at least 20 min in extraction buffer containing (mM); NaCl 100, Tris-HCl [pH 7.5] 10, EDTA 2, 0.5% w/v deoxycholate, 1% v/v triton-X 100, phenylmethylsulfony fluoride 1 mM, $MgCl_2$ 10 mM, 100 IU ml^{-1} aprotinin, sodium orthovanadate 0.1 mM, scraped and transferred to eppendorf tubes and

centrifuged at 12,000 r.p.m. (Sorvall MC12V, Dupont, Boston, MA, U.S.A.) for 5 min. Aliquots were removed for protein assay (Biorad reagent, Biorad, Sydney, Australia) and the extract was then boiled (4 min) in a ratio of 1 : 1 with loading buffer (62.5 mM Tris-HCl [pH 6.8], 20% glycerol, 2% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol, 0.5% bromophenol blue) before being loaded on a 12% SDS-polyacrylamide gel. Equal amounts of protein for each assay condition were separated electrophoretically on 12% SDS-polyacrylamide gels and proteins were transferred onto a nitrocellulose membrane (Hi-Bond C, Amersham, Cardiff, U.K.) for Western analysis. The membrane was blocked in 5% w/v carnation milk/Tris buffered saline/Tween-20 (1% v/v) for 1 h at room temperature and then incubated for 2 h with monoclonal antibodies to either cyclo-oxygenase-1 (ovine) or cyclo-oxygenase-2 (human). Immunoblotted membranes were then incubated with HRP conjugated anti-mouse IgG, and the HRP activity was visualized using an enhanced chemiluminescence kit (Amersham, Cardiff, U.K.).

Materials

All chemicals were of analytical grade or higher. The compounds used and their sources were as follows: dexamethasone (9α-fluoro-16α-methyl-prednisolone), essentially fatty acid-free bovine serum albumin fraction V (BSA), L-glutamine, indomethacin, prostaglandin E_2, thrombin (bovine plasma) (Sigma, U.S.A.); amphotericin B (fungizone), human recombinant basic fibroblast growth factor (Promega, U.S.A.); collagenase type CLS 1, elastase (Worthington Biochemical, U.S.A.); dimethyl sulphoxide, foetal calf serum (Flow Laboratories, Australia); Dulbecco's Modified Eagle's Medium (DMEM) (Flow Laboratories, Scotland); Dulbecco 'A' phosphate buffered saline (PBS) (Oxoid, U.K.); Monomed A, penicillin-G, versene, streptomycin, trypsin (CSL, Australia); PGE_2 antiserum (Upstate Biotechnology Inc., Lake Placid, N.Y., U.S.A.); [6-^{3}H]-thymidine (5 Ci mmol^{-1}), [5,6,8,11,12,14,15 (n)-^{3}H]PGE_2 (183 Ci mmol^{-1}) (Amersham, U.K.); emulsifier safe scintillant (Canberra-Packard, Australia); fluticasone propionate (Glaxo Wellcome, U.K.); anti-smooth muscle α-actin (mouse monoclonal) (M851), monoclonal mouse anti-human endothelial, CD 31 (JC/70A) (M823) (Dako Corporation, U.S.A.); anti-smooth muscle myosin (rabbit polyclonal) (Sigma, U.S.A.); rabbit anti-mouse IgG horseradish peroxidase-conjugated, mouse anti-rabbit IgG horseradish peroxidase-conjugated (Silenus Laboratories, Hawthorn, Australia); cyclo-oxygenase-1 (ovine) monoclonal antibody, cyclo-oxygenase-2 (human) monoclonal antibody (Cayman Chemical, Ann Arbor, MI, U.S.A.).

Stock solutions of dexamethasone (10 mM) and fluticasone propionate (10 mM) were prepared in 100% v/v dimethyl sulphoxide (DMSO). The highest concentration of vehicle DMSO was 0.01% and the threshold for an action of DMSO on ASM DNA synthesis is 0.3%. Stock solutions of indomethacin (10 mM) were prepared in 0.1 M sodium carbonate. Stock solutions of thrombin (300 u ml^{-1}) and bFGF (300 nM) were prepared in 0.25% BSA/PBS. Stock solutions of all other drugs were prepared in distilled water. Intermediate dilutions and final concentrations of drugs were achieved by appropriate dilution with 0.25% BSA-containing DMEM.

Statistical analysis of results

Incubations in experiments in which PGE_2 or [^{3}H]-thymidine uptake was measured were carried out in triplicate and in a minimum of three cell cultures derived from lung resected from heart-lung transplant specimens from separate individuals. Results are presented as grouped data from multiple cultures and are expressed as mean $\pm$ standard error of the mean (s.e.m.); n represents the number of cell cultures. Fold increments were calculated by dividing the response of treated wells by that of the untreated wells on the same 24 well plate. Differences were determined by one-way analysis of variance (ANOVA) after normalization by log transformation followed by Bonferroni test, where appropriate. In some cases, Student's paired t-test was used to determine if there were significant differences between means of pairs. All statistical analyses were performed using Graphpad Prism for Windows (Version 2.01). In all cases, probability levels less than 0.05 ($P<0.05$) were taken to indicate statistical significance.

Results

Incubation with IL-1α and TNF-α inhibits mitogen-induced [^{3}H]-thymidine incorporation

Incubation of smooth muscle with maximally effective concentrations of thrombin (0.3 u ml^{-1}) and bFGF (300 pM) for 28 h (Figure 1) caused a marked increase in [^{3}H]-thymidine incorporation (Figure 2). Similarly, the addition of the combination of IL-1α (0.001 – 0.1 pM) and TNF-α (0.3 – 30 pM) for 52 h evoked an increase in [^{3}H]-thymidine incorporation (Figures 2 and 3). In the presence of the cytokine mixture of IL-1α (0.1 pM) and TNF-α (30 pM) thrombin did not stimulate any further [^{3}H]-thymidine incorporation (Figure 2). Incubation of smooth muscle cells with a mixture of IL-1α (0.01 and 0.1 pM) and TNF-α (3 and 30 pM) for 24 h and then continuously throughout the 28 h exposure to mitogen markedly reduced the increase in [^{3}H]-thymidine incorporation in response to bFGF (Figures 2 and 3).

Dexamethasone inhibits cytokine-mediated inhibition of mitogen-induced [^{3}H]-thymidine incorporation

Dexamethasone (1 – 1000 nM), added 60 min before thrombin or bFGF concentration-dependently inhibited thrombin- or bFGF-stimulated DNA synthesis with a maximum inhibition of 62 $\pm$ 6% and 33 $\pm$ 6%, respectively (Figure 4). In the presence of the cytokine mixture, dexamethasone did not significantly inhibit thrombin-stimulated [^{3}H]-thymidine incorporation (Figure 5). When dexamethasone was added before the cytokines, the interaction of dexamethasone with cytokines and thrombin followed a similar pattern. Thrombin caused a 4.3 $\pm$ 1.4 ($n=5$) fold increase in [^{3}H]-thymidine incorporation which was reduced to 2.3 $\pm$ 0.4 by dexamethasone (100 nM, 25 h before thrombin). In the presence of the cytokine mixture, thrombin did not stimulate any further [^{3}H]-thymidine incorporation (4.8 $\pm$ 0.7, $n=5$) over that induced by the cytokine mixture alone and this was not reduced by dexamethasone (5.9 $\pm$ 1.3 fold increase in [^{3}H]-thymidine incorporation). Paradoxically, dexamethasone caused a concentration-dependent increase in the incorporation of [^{3}H]-thymidine stimulated by bFGF plus the cytokine mixture (Figures 5 and 6). Furthermore, fluticasone propionate (1 nM) had no further inhibitory effect over that produced by the cytokine mixture on bFGF-stimulated [^{3}H]-thymidine incorporation (Figure 7).

In experiments designed to investigate the effects of dexamethasone on cytokine-induced [^{3}H]-thymidine incor-

poration, dexamethasone was added 23 h after the cytokine mixture (and then remained present for the remainder of the experiment, Figure 1) in order to replicate the design of previous studies of the interaction between cytokines, mitogen and glucocorticoids. In four out of five individual experiments, dexamethasone (100 nM) inhibited cytokine-induced [^{3}H]-thymidine incorporation but increased the response to the cytokine mixture in the other culture (Table 1). Analysis of data from the four experiments in which an inhibition was observed, indicated that this effect was statistically significant ($P<0.05$, Student's paired t-test).

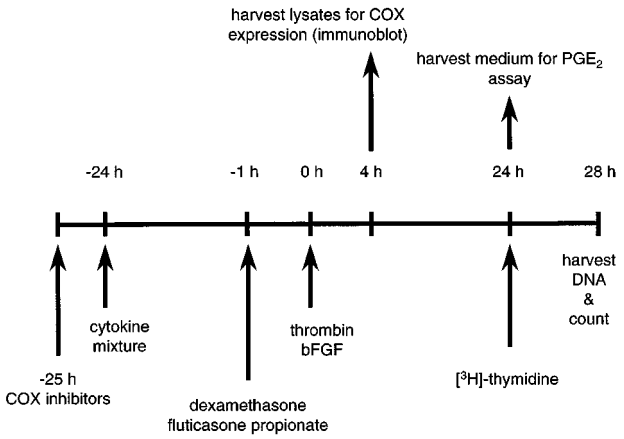

Figure 1 Schematic representation of the experimental protocol. The total mitogen incubation time for measuring effects on DNA synthesis using [^{3}H]-thymidine incorporation was 28 h. Previous studies have established that airway smooth muscle cells enter S phase at 22–24 h (Tomlinson *et al*., 1994). Thus, [^{3}H]-thymidine (1 μCi ml^{-1}) was added at 24 h after mitogen. The cytokine mixture (IL-1α+TNF-α) was added 24 h before the addition of the mitogen (thrombin or bFGF) and then remained present throughout. The cyclo-oxygenase (COX) inhibitors indomethacin or L-745,337 were added 1 h before the cytokine mixture. Dexamethasone or fluticasone propionate were added 1 h before the mitogen. In separate experiments cell lysates were harvested at 4 h after mitogen addition to measure the COX expression which precedes PGE_2 synthesis. Samples of supernatant were taken prior to the addition of [^{3}H]-thymidine for PGE_2 analysis (at 24 h) so that the levels detected could be related to subsequent DNA synthesis.

Production of eicosanoids in cytokine-mediated inhibition of mitogen-stimulated [^{3}H]-thymidine incorporation

Pro-inflammatory cytokines, alone and in various combinations, stimulate the production of a number of eicosanoids

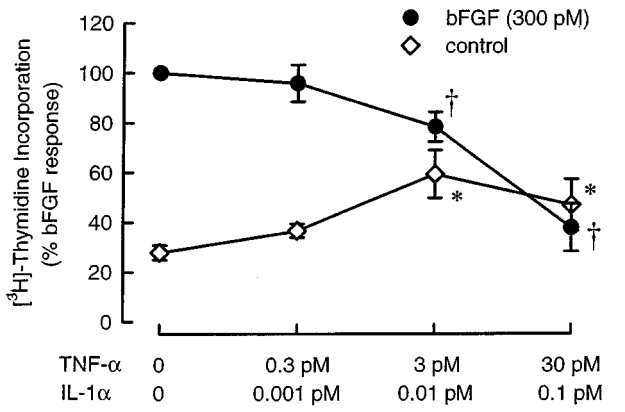

Figure 3 Concentration-effect relationship of the cytokine mixture alone, and in the presence of bFGF, on [^{3}H]-thymidine incorporation. [^{3}H]-Thymidine incorporation is expressed as a percentage of the response to bFGF in the absence of the cytokine mixture. The points plotted represent the grouped means±s.e.mean of five different cell cultures. A significant effect of the cytokine mixture on basal DNA synthesis is indicated by an asterisk (*$P<0.05$, Student's paired t-test). A significant effect of the cytokine mixture on the response to bFGF is indicated by an obelisk (†$P<0.05$, Student's paired t-test).

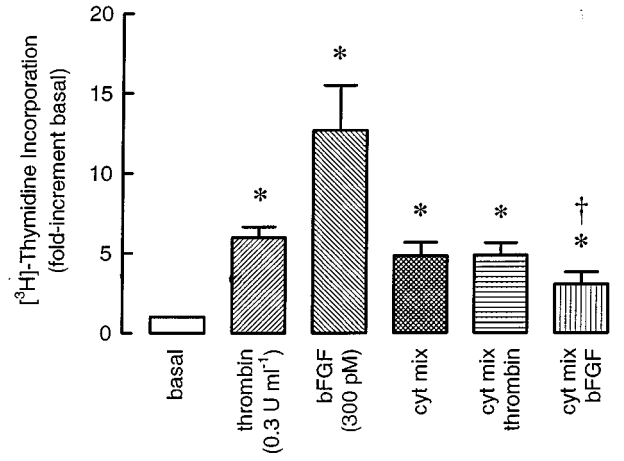

Figure 2 [^{3}H]-Thymidine incorporation in response to mitogens (thrombin, bFGF), a cytokine mixture (cyt mix, IL-1α [0.1 pM]+TNF-α [30 pM]) or a combined stimulation with the cytokine mixture and one of the mitogens. [^{3}H]-Thymidine incorporation is expressed as fold-increments of basal [^{3}H]-thymidine incorporation with basal levels of 2047±374 d.p.m. ($n=7$). Each column represents the grouped means±s.e.mean of five to seven different cell cultures. A significant difference from basal is indicated by an asterisk (*$P<0.05$, ANOVA followed by Bonferroni test). A significant effect of the cytokine mixture on the response to bFGF is indicated by the obelisk (†$P<0.05$, ANOVA followed by Bonferroni test).

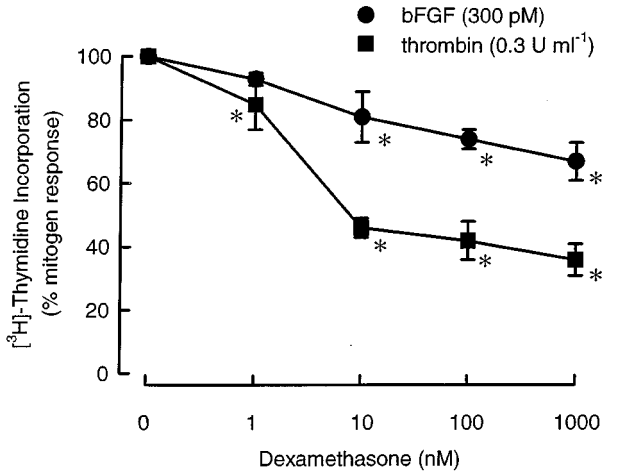

Figure 4 Effect of dexamethasone (1–1000 nM) on incorporation of [^{3}H]-thymidine stimulated by thrombin or bFGF. Cells were serum deprived for 24 h, treated with vehicle (medium) for 24 h at which time medium, thrombin or bFGF was added for a further 28 h period, with [^{3}H]-thymidine (1 μCi ml^{-1}) being present for the final 4 h of the incubation. Dexamethasone was added 1 h before thrombin or bFGF. [^{3}H]-Thymidine incorporation is expressed as a percentage of the response to the mitogen (thrombin, bFGF) in the absence of dexamethasone. The points plotted represent the grouped means±s.e.mean of five different cell cultures. A significant difference from the control mitogen response is indicated by an asterisk (*$P<0.05$, Student's paired t-test).

including PGE_2 and 6-keto-prostaglandin 1α by human cultured airway smooth muscle (Belvisi *et al.*, 1997; Pang & Knox, 1997). Since we and others have shown that PGE_2 inhibits mitogen-stimulated DNA synthesis in human cultured airway smooth muscle (Johnson *et al.*, 1995; Tomlinson *et al.*, 1995), the possibility that cytokine-stimulated PGE_2 may explain the inhibitory effects on mitogen responses was explored. We therefore investigated whether the combination of IL-1α and TNF-α stimulate detectable levels of PGE_2.

Neither unstimulated nor thrombin- or bFGF-stimulated airway smooth muscle produced detectable levels of PGE_2. However, the cytokine mixture, alone or in the presence of thrombin or bFGF, elicited readily detectable levels of PGE_2 (7.7 ± 2.7, 26.1 ± 5.4 and 123.1 ± 38.8 nM; $n = 3$). Dexamethasone (100 nM), the non-selective cyclo-oxygenase inhibitor indomethacin (3 μM) or the selective cyclo-oxygenase-2 inhibitor L-745,337 (0.3 μM) (Chan *et al.*, 1995) reduced PGE_2 levels to below the limit of detection of the assay (0.05 nM).

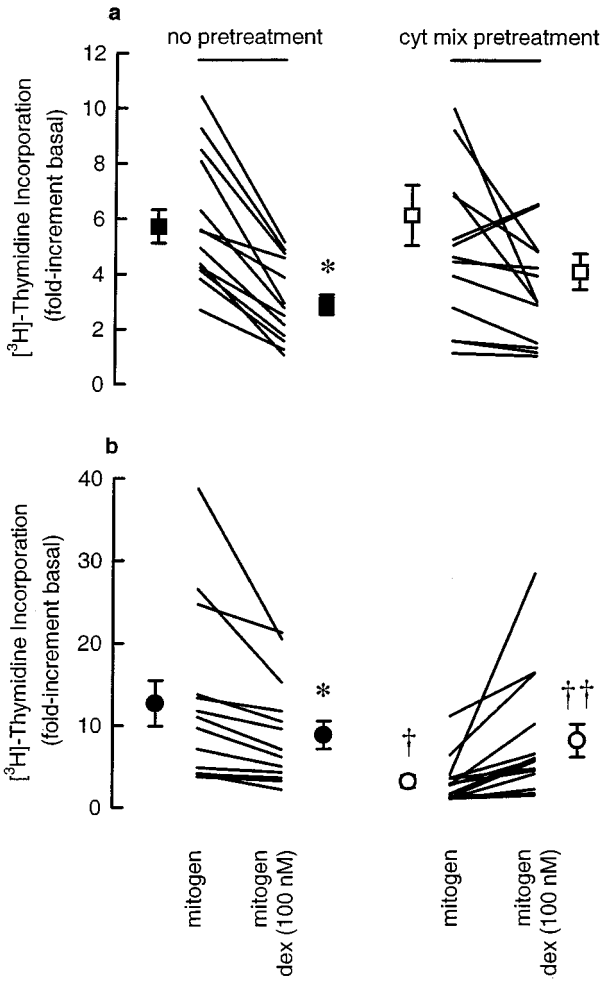

Figure 5 Effect of dexamethasone (dex), in the presence or absence of the cytokine mixture (cyt mix) on incorporation of [^{3}H]-thymidine stimulated by thrombin (a) or bFGF (b). Cells were serum-deprived for 24 h, treated with vehicle (medium) for 24 h or the cytokine mixture (IL-1α [0.1 pM]+TNF-α [30 pM]) at which time medium, thrombin or bFGF was added for a further 28 h period, with [^{3}H]-thymidine (1 μCi ml^{-1}) being present for the final 4 h of the incubation. Dexamethasone (100 nM) was added 1 h before thrombin (0.3 u ml^{-1}) or bFGF (300 pM). [^{3}H]-Thymidine incorporation is expressed as fold-increments of basal [^{3}H]-thymidine incorporation. The lines plotted represent responses in individual experiments and the points with the error bars represent the grouped means$\pm$s.e.mean. *$P<0.05$, Student's paired *t*-test, *c.f.* mitogen response (no pretreatment), †$P<0.05$, Student's paired *t*-test, *c.f.* mitogen (no pretreatment), ††$P<0.05$, Student's paired *t*-test, *c.f.* mitogen/cyt mix pretreatment.

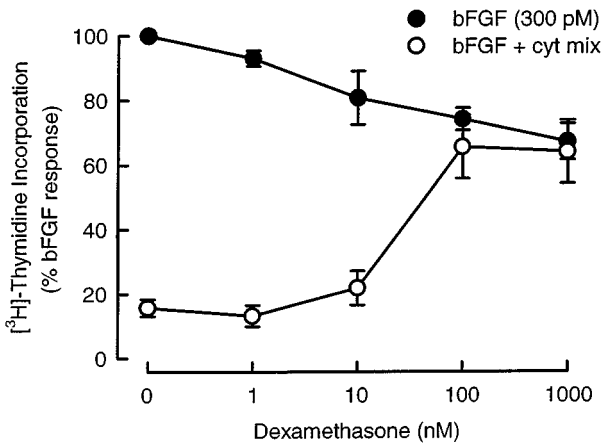

Figure 6 Effect of dexamethasone, in the presence or absence of the cytokine mixture (cyt mix) on incorporation of [^{3}H]-thymidine stimulated bFGF. Cells were serum-deprived for 24 h, treated with vehicle (medium) for 24 h or the cytokine mixture (IL-1α [0.1 pM]+TNF-α [30 pM]) at which time medium or bFGF was added for a further 28 h period, with [^{3}H]-thymidine (1 μCi ml^{-1}) being present for the final 4 h of the incubation. Dexamethasone (1–1000 nM) was added 1 h before bFGF (300 pM). [^{3}H]-Thymidine incorporation is expressed as a percentage of the response to bFGF in the absence of either dexamethasone or the cytokine mixture. The points plotted represent the grouped means$\pm$s.e.mean of seven different cell cultures.

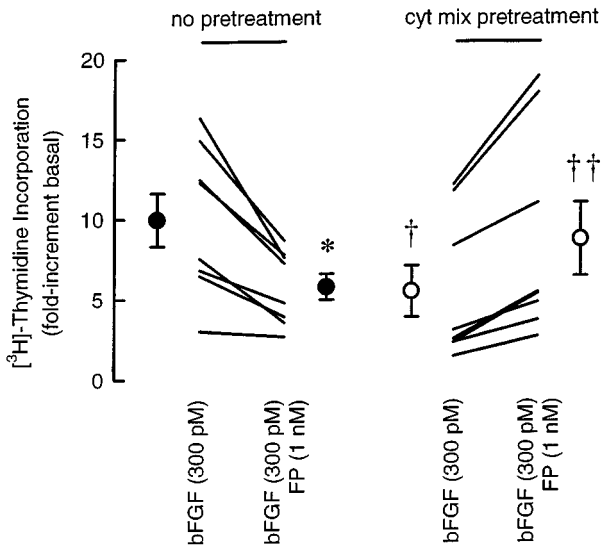

Figure 7 Effect of fluticasone propionate (FP), in the presence or absence of the cytokine mixture (cyt mix) on incorporation of [^{3}H]-thymidine stimulated by bFGF. Cells were serum-deprived for 24 h, treated with vehicle (medium) for 24 h or the cytokine mixture (IL-1α [0.1 pM]+TNF-α [30 pM]) at which time medium, or bFGF was added for a further 28 h period, with [^{3}H]-thymidine (1 μCi ml^{-1}) being present for the final 4 h of the incubation. FP (1 nM) was added 1 h before bFGF (300 pM). [^{3}H]-Thymidine incorporation is expressed as fold-increments of basal [^{3}H]-thymidine incorporation. The lines plotted represent individual experiments and the points with the error bars the means$\pm$s.e.mean of eight different cell cultures. *$P<0.05$, Student's paired *t*-test, *c.f.* bFGF alone, †$P<0.05$, Student's paired *t*-test, *c.f.* mitogen bFGF response (no pretreatment), ††$P<0.05$, Student's paired *t*-test, *c.f.* bFGF/cyt mix response.

Effect of cyclo-oxygenase inhibition on cytokine-mediated inhibition of mitogen-stimulated DNA synthesis

Since indomethacin and L-745,337 inhibited PGE_2 production by cytokine-stimulated airway smooth muscle, we investigated the effects of these cyclo-oxygenase inhibitors on [^{3}H]-thymidine incorporation. Incubation of human airway smooth muscle cells with indomethacin (3 μM) or L-745,337 (0.3 μM) for 53 h had no effect on basal [^{3}H]-thymidine incorporation, nor did they consistently affect the response to thrombin or bFGF (Figures 8 and 9). In contrast, indomethacin or L-745,337 markedly enhanced [^{3}H]-thymidine incorporation in response to the cytokine mixture or to the cytokine mixture plus either thrombin or bFGF (Figures 8 and 9).

In order to remove the confounding influence of glucocorticoids on the production of COX metabolites, indomethacin (3 μM) was added before any other stimuli and remained present throughout. Under these conditions, thrombin caused a 3.6 ± 0.7 fold increase in [^{3}H]-thymidine

Table 1 Effects of dexamethasone (dex) on cytokine mixture (cyt mix)-induced [^{3}H]-thymidine incorporation

	[^{3}H]-Thymidine incorporation (Average, d.p.m.)			
Culture	*Basal*	*Dex* (100 nM)	*Cyt mix*	*Cyt mix + Dex*
1	1738	871	4639	2694
2	1266	1122	9458	5885
3	1223	988	1415	1210
4	923	683	3495	1928
5	414	789	1429	2210

Data are expressed as the average of duplicate incubations in five separate cell cultures. Cytokine mixture = IL-1α (0.1 pM) + TNF-α (30 pM).

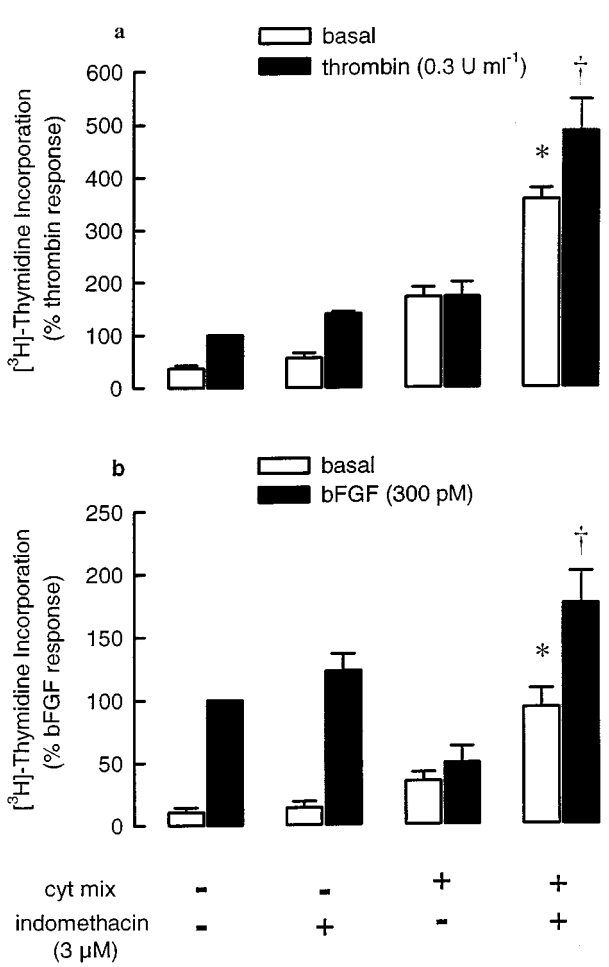

Figure 8 Effects of indomethacin in the presence and absence of the cytokine mixture (cyt mix) on basal and mitogen-stimulated [^{3}H]-thymidine incorporation. Cells were serum-deprived for 24 h, treated with vehicle (medium) or the cytokine mixture (IL-1α [0.1 pM] + TNF-α [30 pM]), for 24 h at which time medium, thrombin (a) or bFGF (b) was added for a further 28 h period, with [^{3}H]-thymidine (1 μCi ml^{-1}) being present for the final 4 h of the incubation. Indomethacin (3 μM) was added 1 h before the addition of the cytokine mixture. [^{3}H]-Thymidine incorporation was expressed as a percentage of the response to thrombin or bFGF in the absence of either indomethacin or the cytokine mixture. Each histogram represents the grouped means $\pm$ s.e.mean of four different cell cultures. A significant effect of indomethacin on the response to the cytokine mixture is indicated by an asterisk ($^*P < 0.05$, ANOVA followed by Bonferroni test). A significant effect of indomethacin on the response to the cytokine mixture plus the mitogen is indicated by the obelisk ($\dagger P < 0.05$, ANOVA followed by Bonferroni test).

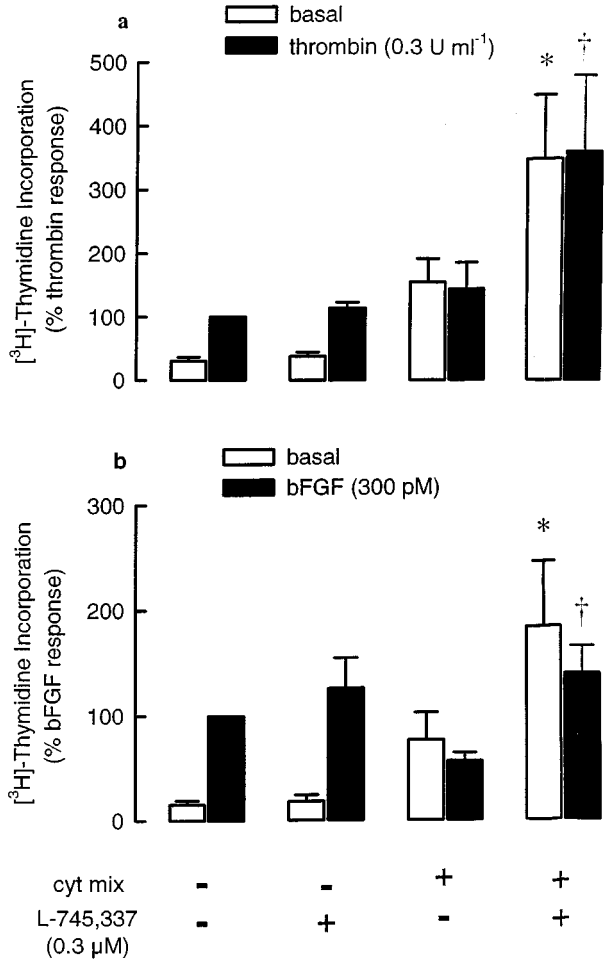

Figure 9 Effects of L-745,337 in the presence of the cytokine mixture (cyt mix) on basal and mitogen-stimulated [^{3}H]-thymidine incorporation. Cells were serum-deprived for 24 h, treated with vehicle (medium) or the cytokine mixture (IL-1α [0.1 pM] + TNF-α [30 pM]), for 24 h at which time medium, thrombin (a) or bFGF (b) was added for a further 28 h period, with [^{3}H]-thymidine (1 μCi ml^{-1}) being present for the final 4 h of the incubation. L-745,337 (0.3 μM) was added 1 h before the addition of the cytokine mixture. [^{3}H]-Thymidine incorporation was expressed as a percentage of the response to thrombin or bFGF in the absence of either L-745,337 or the cytokine mixture. Each column represents the grouped means $\pm$ s.e.mean of three to four different cell cultures. A significant effect of L-745,337 on the response to the cytokine mixture is indicated by an asterisk ($^*P < 0.05$, ANOVA followed by Bonferroni test). A significant effect of L-745,337 on the response to the cytokine mixture plus the mitogen is indicated by the obelisk ($\dagger P < 0.05$, ANOVA followed by Bonferroni test).

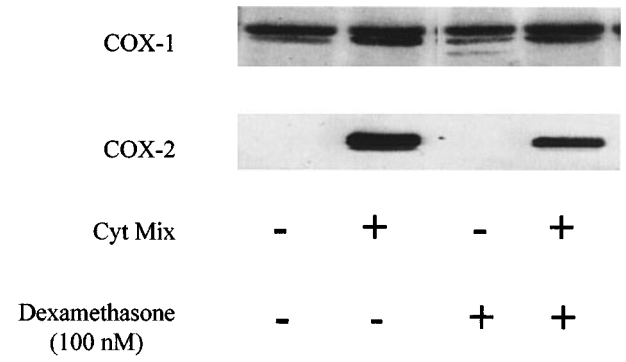

Figure 10 Effects of the cytokine mixture (IL-1α [0.1 pM] + TNF-α [30 pM]) and dexamethasone (100 nM) on the induction of cyclo-oxygenase (COX)-1 and COX-2 protein in human airway smooth muscle cells. Each lane was loaded with 60 μg protein. These blots are representative of similar results obtained in three different cell cultures.

incorporation which was reduced to 1.8 ± 0.3 by dexamethasone (100 nM, added 1 h before thrombin, $n = 10$). The cytokine mixture increased the response to thrombin to 7.7 ± 1.6 ($n = 10$) and this response was reduced by dexamethasone to 3.5 ± 0.5 ($n = 10$). In addition, the direct effects of the cytokine mixture (4.3 ± 1.2 fold increase in [^{3}H]-thymidine incorporation) were significantly reduced by dexamethasone (100 nM) to 2.5 ± 0.5 ($P < 0.05$, Student's paired t-test, $n = 10$).

Cyclo-oxygenase protein expression in human cultured airway smooth muscle

In human airway smooth muscle cells deprived of serum for 28 h COX-1, but not COX-2 protein was detected by Western blotting (Figure 10). However, when the cells were pretreated with the cytokine mixture for 28 h, COX-2 protein was readily detectable and there was no change in the level of COX-1 protein (Figure 10). The cytokine mixture-induced expression of COX-2 protein was attenuated by dexamethasone (100 nM) whereas there was no systematic effect of dexamethasone (100 nM) on COX-1 protein levels (Figure 10).

Discussion

The three major findings in the present study were: (i) cytokine-induced PGE_2 production *via* COX-2 inhibited human cultured airway smooth muscle DNA synthesis; (ii) the paradoxical effect of dexamethasone to increase DNA synthesis stimulated by cytokine/mitogen combinations appears to be explained by the ability of dexamethasone to repress COX-2 induction, decreasing cytokine-induced PGE_2 to concentrations below those that inhibit DNA synthesis; and (iii) the cytokine combination had modest stimulatory effects on DNA synthesis which were inhibited by glucocorticoids and greatly increased by inhibition of COX-2.

Glucocorticoids inhibit airway smooth muscle proliferation and may therefore be beneficial in reducing the airway wall remodelling that occurs in asthma (Schramm *et al*., 1996; Stewart *et al*., 1995a,c; Young *et al*., 1995). Since asthma is a chronic inflammatory disease of the airways in which pro-inflammatory cytokines are present in biologically active amounts in bronchoalveolar lavage fluid aspirated from asthmatic patients (Broide *et al*., 1992; Cembrzynska-Nowak *et al*., 1993; Mattoli *et al*., 1991; Walker *et al*., 1992), we re-evaluated the anti-proliferative effects of the glucocorticoids, dexamethasone and fluticasone propionate (Holliday *et al*., 1994), in the presence of a combination of the cytokines, IL-1α and TNF-α.

Preincubation of smooth muscle cells with the cytokine mixture inhibited the increase in DNA synthesis in response to either bFGF or thrombin, similar to earlier observations made when TNF-α was co-incubated with thrombin, epidermal growth factor or FCS (Stewart *et al*., 1995b). Cytokines such as IL-1α (De *et al*., 1993; 1996) or TNF-α (Amrani *et al*., 1996; Stewart *et al*., 1995b) stimulate DNA synthesis and may therefore contribute to the airway wall remodelling process. The conversion of the cytokine combination from a weak to a strong stimulus for DNA synthesis by COX-2 inhibition is consistent with previous reports in guinea-pig and human airway smooth muscle (De *et al*., 1993; 1996) and human vascular smooth muscle (Libby *et al*., 1988) which indicate that the mitogenic response may be masked by accumulation of growth-inhibitory prostaglandins. Platelet-derived growth factor (PDGF) is reported to have variable effects in human vascular smooth muscle that are explained by a variable expression of COX-2 in cultures from different individuals which in turn produce variable amounts of growth-inhibitory eicosanoids (Bornfeldt *et al*., 1997). PDGF itself does not appear to influence COX-2 induction in this cell type. Our findings suggest that neither thrombin nor bFGF stimulates detectable PGE_2 production, COX inhibition does not have a consistent effect on the magnitude of the DNA synthesis and there is no evidence of a constitutive expression of COX-2 in human cultured airway smooth muscle in this, or in previous studies (Belvisi *et al*., 1997; Pang & Knox, 1997; Vigano *et al*., 1997). Similarly, in human vascular smooth muscle inhibition of COX had no effect on responses to FCS, whereas those to IL-1α were markedly enhanced (Libby *et al*., 1988). The dual effect of the cytokines, namely direct and relatively small stimulation of DNA synthesis and inhibition of DNA synthesis elicited by efficacious mitogens such as bFGF, is similar to the profile of activity of transforming growth factor β (TGFβ) (Cohen *et al*., 1997). It remains to be determined whether there is any overlap in the signalling pathways used by TGFβ and the cytokine mixture.

We confirmed the inhibitory effects of dexamethasone on thrombin and cytokine-stimulated DNA synthesis, but the effects of bFGF were inhibited to a lesser, albeit significant extent. There is an apparent conflict between the modest clinical impact of the glucocorticoids on airway hyperresponsiveness in asthma (Barnes, 1990) and the premise that airway wall remodelling (due in part to proliferation of airway smooth muscle) accounts for a major part of the airway hyperresponsiveness (Pare & Bai, 1995), as it would be predicted that the glucocorticoid inhibition of airway smooth muscle proliferation would reverse this and possibly other aspects of airway wall remodelling (Olivieri *et al*., 1997). The observation that mitogens are differentially sensitive to the inhibitory effects of glucocorticoids may indicate that airway smooth muscle proliferation is not optimally controlled by this class of anti-asthma agents.

Paradoxically, dexamethasone increased DNA synthesis in airway smooth muscle cells exposed to cytokines and bFGF. A slightly different pattern of interaction was observed in experiments where thrombin was the mitogen, probably because dexamethasone had a more pronounced inhibitory effect and the direct stimulation by thrombin itself was significantly less than that of bFGF. Nevertheless, DNA synthesis in the presence of cytokine/thrombin combination was not inhibited by dexamethasone. One interpretation of these unexpected findings is that glucocorticoids inhibit the

cytokine-mediated suppression of the mitogenic response. Amongst the potential mechanisms of this paradoxical action of dexamethasone is inhibition of the eicosanoid pathway, since recent studies indicate that a variety of cytokines can stimulate the production of PGE_2, which inhibits airway smooth muscle proliferation (Johnson *et al*., 1995; Tomlinson *et al*., 1995). Dexamethasone represses the induction of COX-2 and consequently inhibits the cytokine-stimulated PGE_2 production (Belvisi *et al*., 1997; Pang & Knox, 1997). An involvement of eicosanoids in the effects of TNF-α was discounted in our previous study since the suppressive effects of TNF-α alone were not influenced by the COX inhibitor, indomethacin and no eicosanoid synthesis was detectable (Stewart *et al*., 1995b). However, the present evidence clearly implicates PGE_2 in the inhibitory effects of the cytokine combination. The cytokine mixture induced levels of PGE_2 (7–130 nM) that are maximal for inhibition of mitogen-stimulated DNA synthesis (Gillzan & Stewart, unpublished observations). The lack of PGE_2 production basally or in the presence of thrombin or bFGF, despite detection of COX-1 by Western blotting, suggests that the level of arachidonic acid mobilization was insufficient for PGE_2 levels to reach assay detection limits (0.05 nM). There was synergy between the cytokines and the mitogens in PGE_2 production, but not in COX-2 induction (data not shown), suggesting that the site of the synergistic action on PGE_2 production is at the level of arachidonic acid mobilization. Further work is in progress to elucidate the mechanism of this synergy. The lack of involvement of the COX pathway in the effects of TNF-α (Stewart *et al*., 1995b) suggests that it may be the IL-1α in the combination that induces COX-2, as is suggested by the findings of Pang & Knox (1997).

The induction of PGE_2 production by cytokines was further implicated in the cytokine-mediated inhibition of DNA synthesis by experiments in which COX-2 activity was reduced by: (a) the non-selective COX inhibitor, indomethacin; (b) the COX-2 selective inhibitor L-745,337; and (c) dexamethasone which represses COX-2 induction. The activity of each of these interventions was confirmed by radioimmunoassay of PGE_2 which was reduced to less than 0.05 nM – concentrations of PGE_2 which have no effect on DNA synthesis (Gillzan & Stewart, unpublished observations). In addition, dexamethasone inhibited cytokine-mediated induction of COX-2 protein without influencing COX-1 protein levels, consistent with earlier reports of the effect of glucocorticoids on different cytokines and their combinations (Belvisi *et al*., 1997; Pang & Knox, 1997). The most important evidence implicating PGE_2 or another inhibitory eicosanoid is drawn from the contrast between the effects of indomethacin, L-745,337 and dexamethasone on DNA synthesis induced by mitogens or by cytokine and cytokine/mitogen combinations. Neither COX inhibitor influenced DNA synthesis in response to mitogens and dexamethasone was inhibitory, albeit to different extents with thrombin or bFGF. However, each of the COX inhibitors enhanced DNA synthesis in response to the cytokines and the cytokine/mitogen combination. Whilst the interactions with dexamethasone were more complicated, it was clear that this glucocorticoid markedly inhibited the direct stimulatory effects of the cytokine combination when COX activity was blocked, consistent with our previous observations on the effects of TNF-α alone (Stewart *et al*., 1995b) which does not appear to significantly increase eicosanoid production (Pang & Knox, 1997; Stewart *et al*., 1995b). Moreover, in cultures not treated with a COX inhibitor, in four of five experiments the glucocorticoid significantly reduced cytokine-stimulated DNA synthesis. The variability in this latter experiment between cultures may relate to a variable importance of the regulatory effects of eicosanoid production and mitogenesis inhibition by glucocorticoids. Despite this direct inhibition of both cytokine and mitogen responses, dexamethasone increased DNA synthesis when added prior to the cytokine/mitogen combination. In the case of bFGF/cytokine combinations, dexamethasone and fluticasone propionate returned the level of DNA synthesis to those observed when bFGF responses (in the absence of cytokines) were maximally inhibited by these glucocorticoids. Thus, the dexamethasone response could be viewed as a reversal of the cytokine inhibitory effect. It is noteworthy that when airway smooth muscle cultures were pretreated with indomethacin, the cytokine suppressive effects were prevented and dexamethasone inhibited, rather than increased DNA synthesis. Protocols which examined the importance of the order of dexamethasone addition on [^{3}H]-thymidine incorporation indicated that the pattern of dexamethasone/cytokine mixture/mitogen response described above was similar, regardless of whether dexamethasone was introduced 1 h before the cytokine mixture or 1 h before the mitogen. Furthermore, the similarity between the effects of fluticasone propionate and dexamethasone suggests that the cytokine/glucocorticoid interaction is a general effect of this class of agents.

A number of functions of PGE_2 other than direct inhibition of smooth muscle cell cycle progression should be considered. There have been several studies indicating that IL-1α impairs relaxant responses of airway smooth muscle to β_2-adrenoceptor stimulants and more recently a similar inhibitory action was reported for PGE_2 (Shore *et al*., 1997). The mechanism of the uncoupling of β_2-adrenoceptors and PGE_2 (EP) receptors from adenylate cyclase has not been established, but COX activity has been implicated in β_2-adrenoceptor desensitization (Daffonchio *et al*., 1985). In considering the impact of IL-1α exposure on airway smooth muscle in the asthmatic airway, predicting the outcome for remodelling is complicated by the interrelated phenomena which are elicited by IL-1α. Thus, whilst the cytokine induction of PGE_2 is growth inhibitory, it may be that the heterologous desensitization of β_2-adrenoceptors by PGE_2 is of more significance to the regulation of airway smooth muscle proliferation in asthma, since β_2-adrenoceptor agonists partially inhibit airway smooth muscle proliferation (Schramm *et al*., 1996; Tomlinson *et al*., 1994; Young *et al*., 1995). Glucocorticoids reverse the desensitization of β_2-adrenoceptors in airway smooth muscle (Hauck *et al*., 1997) and in non-muscle cell types (Chong *et al*., 1997). The latter actions of glucocorticoids may be of greater consequence than any effect of limiting PGE_2 production on airway wall remodelling in patients treated with inhaled β_2-adrenoceptor agonists. Additional functions of PGE_2 include regulation of actin isoform expression (Li *et al*., 1997) and modulation of cytokine release (Williams & Shacter, 1997), which is of increasing interest in relation to airway smooth muscle cells which are now recognized as a source of multiple cytokines (Pang & Knox, 1997). The direct and indirect effects of glucocorticoids on PGE_2-dependent actions of cytokines warrants further attention.

In conclusion, our results suggest that dexamethasone prevents the inhibitory effect of cytokines on mitogen-stimulated DNA synthesis by repressing the induction of COX-2 which in turn is responsible for the production of anti-mitogenic prostanoids. Whilst it is difficult to extrapolate these findings to the *in vivo* use of glucocorticoids in asthma, our observations raise the possibility that some beneficial effects of glucocorticoids on the airway wall remodelling process may be limited by repression of COX-2.

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