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The effect of glucocorticoids on proliferation of human cultured airway smooth muscle

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1 Airway smooth muscle proliferation is a significant component of the airway wall remodelling that occurs in asthma. In this study, the effects of glucocorticoids on mitogenic responses of human airway smooth muscle have been examined.

2 Pretreatment of smooth muscle cells with dexamethasone (100 nM, 60 min) inhibited thrombin-induced increases in [^{3}H]-thymidine incorporation (DNA synthesis) and cell number.

3 Inhibition of thrombin-induced [^{3}H]-thymidine incorporation was also observed with hydrocortisone (0.01-1 μM) and methylprednisolone (0.001-0.1 μM) pretreatment. In contrast, pretreatment with either testosterone (0.001-1 μM), progesterone (0.001-1 μM), 17β-oestradiol (0.001-1 μM), or aldosterone (0.001-1 μM) had no effect on the response to thrombin.

4 Responses to a range of mitogens including thrombin (0.01-10 u ml^{-1}), epidermal growth factor (EGF, 3-3000 pM), basic fibroblast growth factor (bFGF, 0.3-300 pM) and foetal calf serum (FCS, 0.1-10% v/v) were inhibited by dexamethasone (100 nM) pretreatment. However, the magnitude of the inhibitory effect was dependent on the mitogen, with EGF being the least, and thrombin being the most sensitive to the inhibitory effect.

5 The potency of hydrocortisone as an inhibitor of [^{3}H]-thymidine incorporation was reduced when FCS (10% v/v, which caused a 40 fold increase in [^{3}H]-thymidine incorporation) was used as the mitogen in place of thrombin (0.3 u ml^{-1}, which caused a 10 fold increase in [^{3}H]-thymidine incorporation).

6 The effect of post-treatment with dexamethasone (100 nM) indicated that addition of the glucocorticoid up to 17-19 h after thrombin (0.3 u ml^{-1}) produced similar degrees of inhibition to those obtained when it was added as a pretreatment. Dexamethasone no longer produced an inhibitory effect if added 21 h or more after the addition of thrombin.

7 These results suggest that glucocorticoids regulate airway smooth muscle proliferation initiated by a range of stimuli. This effect may be of importance in the therapeutic actions of these compounds in asthma, particularly when they are used for prolonged periods of time.

Keywords: Airway smooth muscle; dexamethasone; methylprednisolone; hydrocortisone; proliferation; asthma; growth factors

Introduction

Investigations of the pathogenesis of asthma have recently been focused on the inflammatory aspects of this disease (Morley, 1993). One manifestation of the airway inflammation in asthma is an increase in airway hyperresponsiveness, the exaggerated airway narrowing provoked by a variety of unrelated stimuli (Chung, 1990). Airway hyperresponsiveness remains poorly understood despite intensive investigation. However, interest is growing steadily in the possibility that a tissue remodelling process in the airway wall plays an important part in the development of this phenomenon (Hirst & Twort, 1992; Stewart *et al.*, 1993; 1995). Recent *post mortem* morphometric studies from airways of asthmatic and non-asthmatic, non-atopic controls, using mathematical modelling indicate that there is sufficient thickening of the airway wall in asthmatic patients to explain a major part of the airway hyperresponsiveness in asthma (James *et al.*, 1989; Wiggs *et al.*, 1992). In a thickened airway wall a given amount of shortening of airway smooth muscle causes a larger increase in airway resistance than that observed in a healthy airway of normal dimensions. There have been few studies investigating the nature of the thickening, but those to date suggest that both hypertrophy and hyperplasia of the airway smooth muscle contribute to the thickening (Heard & Hossain, 1971; Hossain, 1973; Ebina *et al.*, 1990; 1993). In addition, there is little known about the mechanism of the induction of the remodelling process, the effect of anti-asthma drugs or whether the remodelling is reversible.

Several laboratories have examined potential proliferative stimuli using airway smooth muscle cultured from the large airways of a number of species, including man. Thus, established mitogens for cultured airway smooth muscle include: histamine (Panettieri *et al.*, 1990); interleukin-1β (IL1β) (De *et al.*, 1993); hexosaminidase (Lew & Rattazi, 1991); thromboxane A_2 (Noveral *et al.*, 1992; Stewart *et al.*, 1992); endothelin-1 (Noveral & Grunstein, 1992; Stewart *et al.*, 1994); platelet-derived growth factor (PDGF) (Hirst *et al.*, 1992; Stewart *et al.*, 1992); thrombin (Tomlinson *et al.*, 1994); and epidermal growth factor (EGF) (Stewart *et al.*, 1992).

The effects of anti-asthma agents on airway smooth muscle proliferation have yet to be systematically characterized. We have shown that the selective β_2-adrenoceptor agonist, salbutamol, inhibits proliferative responses to a range of mitogens, suggesting a functional antagonism of the proliferative response in a manner similar to its ability to antagonize functionally the actions of a range of bronchoconstrictor substances (Tomlinson *et al.*, 1994). Furthermore, the anti-proliferative effect of β_2-adrenoceptor agonists is at least partly a result of elevation of adenosine 3':5'-cyclic monophosphate (cyclic AMP), since it is mimicked by cyclic AMP analogues and other agents which elevate cyclic AMP, such as prostaglandin E_2, and is inhibited by inhibition of protein kinase A activation (Tomlinson *et al.*, 1995). The phosphodiesterase inhibitor, theophylline, also has inhibitory effects on proliferation of guinea-pig cultured airway smooth muscle (Stewart *et al.*, 1992).

Glucocorticoids act primarily as anti-inflammatory agents in asthma and partially reduce the characteristic airway hy-

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perresponsiveness in this disease (Sotomayor *et al.*, 1984). Comparative clinical trials indicate that there is a better outcome in terms of lung function when inhaled steroids are used in place of β_2-selective agonists as the first-line treatment of newly diagnosed asthma (Haahtela *et al.*, 1994). The glucocorticoids have many potential anti-inflammatory effects that could be of importance to their anti-asthma action. These anti-inflammatory actions result from inhibition of inflammatory mediator synthesis and release, including that of the cytokines and low molecular weight mediators derived from the activity of phospholipase A_2 on membrane phospholipids (Barnes & Pedersen, 1993). These drugs also reduce the number of eosinophils and activated lymphocytes in the airways (Boschetto *et al.*, 1987) and decrease microvascular leakage from the bronchial circulation (Erjefalt & Persson, 1986).

In the present study, we have investigated the effects of glucocorticoids on airway smooth muscle proliferation induced by a range of mitogens including thrombin, foetal calf serum (FCS), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Our results indicate that glucocorticoids are potent inhibitors of airway smooth muscle proliferation and that endogenous levels of glucocorticoids have the potential to modulate proliferative responses.

Methods

Cell culture

Bronchial airway smooth muscle was obtained from macroscopically normal lung resection specimens provided by the anatomical pathologists at The Royal Melbourne and Alfred Hospitals (Melbourne). Cultures were prepared as previously described in detail (Tomlinson *et al.*, 1994). Briefly, the tissue was partially digested in Dulbecco's Modified Eagle's Medium (DMEM), (supplemented with 2 mM L-glutamine, 100 μg ml^{-1} streptomycin, 100 u ml^{-1} penicillin-G, 2 μg ml^{-1} amphotericin B and 0.25% w/v bovine serum albumin, BSA) containing 3 mg ml^{-1} collagenase for 30 min at 37°C, and approximately 0.5 g smooth muscle was further digested by a 2 h incubation in 0.5 mg ml^{-1} elastase, followed by an 18 h incubation in collagenase (3 mg ml^{-1}) at 37°C. Cell suspensions were centrifuged (10 min, 100 *g*, 25°C), washed three times in supplemented DMEM, resuspended in 25 ml DMEM containing 10% v/v heat-inactivated foetal calf serum (FCS), seeded into 25 cm^2 Falcon culture flasks and incubated (37°C, 5% CO_2) for 7 to 10 days until monolayer confluency was reached. Cells were then harvested weekly by 10 min exposure to 0.5% trypsin, 1 mM EDTA and passaged at a 1:3 split ratio into 75 cm^2 Falcon culture flasks. Cells at passage numbers 3 to 15 were used for experiments.

Immunocytochemistry

Cells were subcultured into 8-well glass tissue culture chamber slides, and grown to 100% confluency in DMEM (10% FCS). Slides were washed three times in PBS, before fixation for 7 min in ice-cold acetone and stored for up to 4 weeks at 4°C before staining. Following rehydration in PBS/BSA 0.25% for 20 min, the cells were incubated in the primary antibody for 60 min at 22°C, which was removed by washing and exposed to the secondary antibody (goat anti-mouse Ig F(ab')2 fragment or goat anti-rabbit IgG which were either FITC- or horseradish peroxidase (HRP)-conjugated), for 60 min at 22°C. Controls were provided by substituting the primary antibody with PBS/BSA 0.25%. The staining of the fixed cells was analysed by fluorescence (Olympus BH2 microscope equipped with fluorescence light source) or light microscopy (when second antibody was HRP-conjugated). The characteristics of the antibodies used to identify the smooth muscle in culture were established on native airway wall specimens (of the same dimensions as those used for generating airway smooth muscle cultures) that had been fixed by immersion in buffered formalin saline and subsequently embedded in paraffin. The antibodies used were raised against smooth muscle α-actin, smooth muscle myosin, and PECAM-1 (CD31) which is a marker of endothelial cells.

The expression of smooth muscle α-actin and smooth muscle myosin was observed in all cultures used in this study. These cultures did not express detectable PECAM-1 staining. Paraffin-embedded sections of the airway adjacent to that used for generation of cultures stained positively for smooth muscle α-actin and myosin in bundles of airway smooth muscle and blood vessels only and the antibody against PECAM-1 stained vascular endothelium, confirming the specificity of these antibodies for the target antigens.

Proliferation assay

Cells were subcultured into 24 well plates at a 1:3 ratio and allowed to grow to monolayer confluency over a 72–96 h period in an atmosphere of 5% CO_2 in air at 37°C. The serum-containing media was replaced with serum-free DMEM for a 24 h period to produce growth arrest. In some experiments, the cells were pretreated with glucocorticoids 60 min before the addition of mitogen. The stimulant (mitogen) was added to the appropriate wells together with a supplement containing insulin, transferrin, and selenium (Monomed A, 1% v/v). Monomed A was added to provide progression factors which are essential for the mitogenic activity of growth factors such as thrombin, EGF and bFGF (Stewart *et al.*, 1994; 1995). Mitogens and inhibitors (glucocorticoids) were left in contact with cells from the time of addition until the end of the experiment. Cells were incubated for 24 h (37°C, 5% CO_2) before being pulsed with [^{3}H]-thymidine (1 μCi ml^{-1} for 4 h), or 48 h before being pulsed with [^{3}H]-leucine (1 μCi ml^{-1} for 4 h) to measure incorporation of radiolabel into newly synthesized DNA and protein, respectively, according to our previous study (Tomlinson *et al.*, 1994). Radioactivity was determined by liquid scintillation counting (Packard Tri-Carb 4640). In separate experiments cell number was determined by counting cells in a haemocytometer after 48–72 h incubations under the conditions described above. Cells were removed from each of the wells of 6 well culture plates used in these experiments by exposure to 200 μl of 0.5% trypsin in PBS containing 1 mM EDTA for a period of 30–45 min to ensure that the cells were completely dissociated from each other and from the culture plate to enable an accurate count to be made. At the end of this period, a further 200 μl of PBS (20% FCS) was added to prevent cell lysis by trypsin and cells were counted directly in a haemocytometer. Protein synthesis and cell number measurements were made after a minimum incubation period of 48 h to ensure that there had been sufficient time for cell division to take place.

Entry of cells into S-phase

The onset of S-phase in the cell cycle is defined by the point at which the cells begin to synthesize DNA. In experiments to determine the duration of G_1 (the time between the addition of mitogen and the beginning of DNA synthesis) 1 h exposures of separate groups of cells to [^{3}H]-thymidine were carried out at 2 hourly intervals from 0 to 34 h after the addition of thrombin. The amount of [^{3}H]-thymidine incorporated during the 1 h pulses was determined as described above. The time point at which the level of [^{3}H]-thymidine incorporation increased significantly above the baseline level marks the beginning of S-phase.

Assessment of cytotoxicity

The possible cytotoxicity of dexamethasone was assessed by cell counting, determination of protein synthesis, exclusion of trypan blue and light microscopic inspection of the cells. To determine trypan blue exclusion after a 24 h incubation in dexamethasone, trypan blue was added to the medium to

achieve a final concentration of 0.2% (w/v). After a 5 min incubation period at 37°C, the medium was aspirated and replaced with phenol red-free medium. Four high powered fields (magnification 200×) were inspected per well and 4 wells were used for each treatment. Because the cells grow in multilayers it is not possible to determine the total cell number when the cells are adherent, therefore the number of non-viable cells per high powered field is presented.

Statistical analyses

Each treatment in an individual experiment was carried out in quadruplicate. Each experiment was usually performed in at least three different cultures obtained from three different individuals. Results are presented as grouped data from multiple cultures and are expressed as mean ± s.e. of n cultures. Fold increments were calculated by dividing the response of treated wells by that of the control wells on the same 24 well plate. The grouped data was analysed by paired t test after normalisation by log transformation. The Bonferroni adjustment for multiple comparisons was used when necessary. Log concentration-response curves were analysed by ANOVA and individual differences were then identified with Fisher's LSD. Differences were considered to be significant when $P<0.05$.

Materials

All chemicals used were of analytical grade or higher. The compounds used and their sources were as follows: (+)-aldosterone, L-glutamine, essentially fatty acid free bovine serum albumin fraction V (BSA), dexamethasone (9α-fluoro-16α-methylprednisolone), hydrocortisone (11β,17α,21-trihydroxypregn-4-ene-3,20-dione), 17β-oestradiol, methylprednisolone (6α-methyl-11β,17α,21-trihydroxy-1,4-pregnadione-3,20-dione), progesterone, testosterone, thrombin (bovine plasma), human recombinant [Leu^{21}]-epidermal growth factor (EGF), Sigma, U.S.A.; amphotericin B (Fungizone), human recombinant basic FGF (bFGF), Promega, U.S.A.; collagenase type CLS 1, elastase, Worthington Biochemical, U.S.A.; Dulbecco 'A' phosphate buffer saline (PBS), Oxoid, England; trypsin, versene, penicillin-G, streptomycin, Monomed A, CSL, Australia; dimethyl sulphoxide (DMSO), trichloroacetic acid (TCA), foetal calf serum (FCS), Flow Laboratories, Australia; Dulbecco's Modified Eagle's Medium (DMEM), Flow Laboratories, Scotland. [6-^{3}H]-thymidine (185 GBq mmol^{-1}, 5 Ci mmol^{-1}), Amersham, U.K.; L-[4,5-^{3}H(N)]-leucine (2.0 TBqmmol^{-1}, 54 Ci mmol^{-1}) ICN Radiochemicals, U.S.A.; emulsifier-safe scintillant, Canberra-Packard, Australia.

The antibodies used for immunocytochemistry were anti-smooth muscle α-actin (mouse monoclonal) (Dako M851), monoclonal mouse anti-PECAM-1 (DAKO-CD31, JC/70A) (Dako M823), Dako Corporation, U.S.A.; anti-mouse Ig F(ab')2 fragment FITC-conjugate (host sheep), sheep anti-rabbit Ig HRP-conjugate (Silenus DDAF), Silenus, Australia, and anti-smooth muscle myosin (rabbit polyclonal), provided by Prof. M. Sparrow, Perth, W. Australia.

Results

Effect of dexamethasone treatment on airway smooth muscle proliferation

Previous studies have established that 0.3–1.0 u ml^{-1} thrombin stimulates a maximal or near maximal proliferative response in human cultured airway smooth muscle (Tomlinson *et al.*, 1994). In the present study, 0.3 u ml^{-1} thrombin stimulated an 11.4 fold increase in incorporation of [^{3}H]-thymidine and a 1.22 fold increase in cell number (Figure 1). Pretreatment with dexamethasone (100 nM) for 60 min before exposure to thrombin and then continuously throughout the experiment (28 h for [^{3}H]-thymidine) significantly reduced [^{3}H]-thymidine incorporation. The increases in cell number in response to either thrombin or FCS were prevented by pretreatment with dexamethasone (100 nM). There was no evidence of cytotoxicity of dexamethasone (100 nM), since cell number in unstimulated wells was not affected nor were there any morphological changes detectable at the light microscopic level. Furthermore, protein synthesis as measured by [^{3}H]-leucine incorporation, was not affected by 48 h incubation of unstimulated cells with dexamethasone (dexamethasone pretreatment = 99 ± 5% of control [^{3}H]-leucine incorporation, $n=5$). In two cultures, incubation with dexamethasone (100 nM) for 24 h had no effect on the number of cells that took up trypan blue (between 0 and 4 cells per high powered field, approximate cell number in the area inspected is 1000).

Concentration-dependence of the inhibitory effect of dexamethasone, methylprednisolone and hydrocortisone on thrombin-stimulated DNA synthesis

Dexamethasone (10^{-10}–10^{-5} M) significantly ($P<0.05$, ANOVA) inhibited thrombin (0.3 u ml^{-1})-stimulated [^{3}H]-thymidine incorporation (Figure 2), with a threshold concentration of between 10^{-10} and 10^{-9} M and a maximum inhibitory effect between 10^{-9} and 10^{-8} M. The effect of methylprednisolone or hydrocortisone on thrombin-stimulated mitogenesis was examined to ascertain whether the actions of dexamethasone were restricted to this particular glucocorticoid. Hydrocortisone (10^{-8} M–10^{-5} M, 60 min pretreatment) and methylprednisolone (10^{-9}–10^{-6} M, 60 min pretreatment), each significantly ($P<0.05$, ANOVA) inhibited the (0.3 u ml^{-1}) thrombin-stimulated [^{3}H]-thymidine incorporation (Figure 2). Methylprednisolone and dexamethasone were equipotent and significantly ($P<0.05$, Fisher's LSD) more potent than hydrocortisone.

Dependence of glucocorticoid inhibition of DNA synthesis on the mitogen and its concentration

Pretreatment with a maximally effective concentration of dexamethasone (100 nM) inhibited [^{3}H]-thymidine incorporation in cells treated with a range of thrombin concentrations (0.1–10 u ml^{-1}) by approximately 70% at each concentration (Figure 3a). Dexamethasone (100 nM) pretreatment also inhibited the incorporation of [^{3}H]-thymidine in cells stimulated by EGF (3–3000 pM), bFGF (0.3–300 pM), and FCS (0.3–10% v/v) (Figure 3b–d), although the level of inhibition ranged from 71% in the case of thrombin (10 u ml^{-1}) to 37% in the case of EGF (3 nM). Thus, EGF is significantly ($P<0.05$, Student's paired t test) less sensitive to inhibition by dexamethasone than thrombin. The concentration-response curve for inhibition by hydrocortisone of the response to thrombin (Figure 2) suggested that the concentration of this steroid which produced a maximal inhibitory effect was within the range (100–500 nM) that has been detected in the plasma of healthy subjects and asthmatics (Ditzel *et al.*, 1964; Nicolaizik *et al.*, 1994). Further experiments were carried out to establish the effect of hydrocortisone against a more efficacous mitogenic stimulus to determine whether higher concentrations of the steroid were required for inhibition under such conditions. The fold increment in [^{3}H]-thymidine incorporation induced by FCS (10%) 39.6 ± 6.4 was significantly ($P<0.05$, Student's unpaired t test) greater than that induced by thrombin (0.3 u ml^{-1}) 9.5 ± 3.0. On this basis, FCS (10%) was used as the more efficacious stimulant of proliferation. The inhibitory effect of hydrocortisone on the response to FCS (10%) was significantly ($P<0.05$, ANOVA) less than that on the response to thrombin (0.3 u ml^{-1}) (Figure 4).

Effects of non-glucocorticoid steroids on thrombin-stimulated DNA synthesis

Airway smooth muscle was pretreated separately with a number of steroids lacking glucocorticoid activity to determine whether the inhibitory effects of the glucocorticoids were lim-

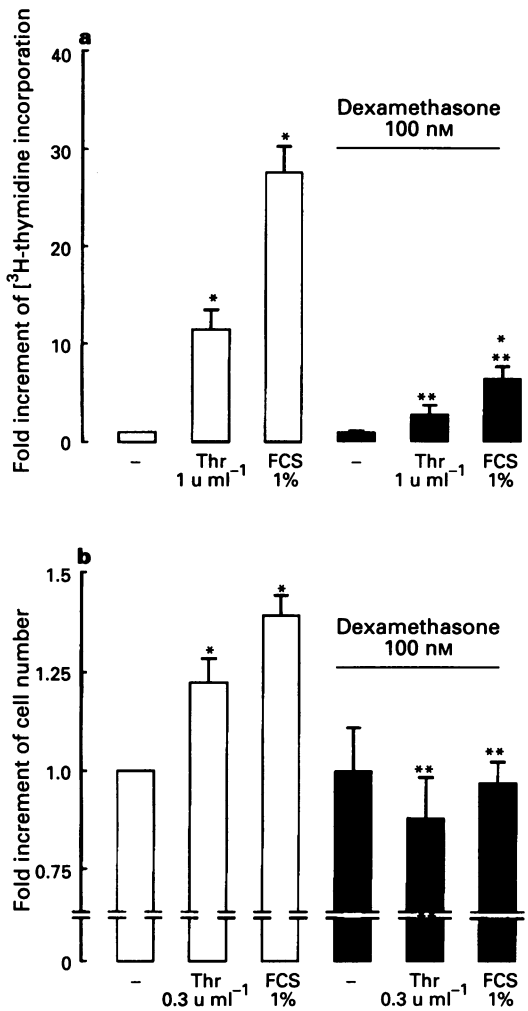

Figure 1 Effect of dexamethasone (100 nM), (solid columns) pretreatment on (a) the incorporation of [^{3}H]-thymidine and (b) increases in cell number in thrombin (0.3 u ml^{-1}) or FCS (1% v/v)-treated airway smooth muscle. Results are expressed as fold increments over the unstimulated (−) level of the [^{3}H]-thymidine incorporation (a, unstimulated incorporation of [^{3}H]-thymidine ranged from 385±67 to 792±98 d.p.m. in quadruplicate incubations) or cell number (b, unstimulated cell number $2.14\pm0.77\times10^4$ cells per well), and are presented as the means and s.e.mean of at least 3 experiments in three different cultures. *$P<0.05$, Student's paired t test, comparing the mitogen-treated cells with the corresponding controls (−). **$P<0.05$, Student's paired t test, comparing the response in the presence of dexamethasone (100 nM) with that in its absence.

ited to this class of steroids. At concentrations of 1 to 1000 nM, testosterone, progesterone, 17β-oestradiol or aldosterone had no significant effect ($P>0.05$, paired Student's t test) on the thrombin (0.3 u ml^{-1})-induced increase in [^{3}H]-thymidine incorporation (Table 1).

Time-course study of dexamethasone inhibition of thrombin-mediated DNA synthesis

Separate experiments were carried out to determine the time point of entry into the S phase by the airway smooth muscle cells following stimulation with thrombin (0.3 u ml^{-1}). Cells were stimulated with thrombin (0.3 u ml^{-1}) for various times and then pulsed for 1 h with [^{3}H]-thymidine. Incorporation of [^{3}H]-thymidine increased significantly ($P<0.05$, Student's paired t test) above the baseline level at 22 h with a plateau level of incorporation occurring at 30 h after thrombin (0.3 u ml^{-1}) stimulation ($n=3$ cell lines, each line being from a different individual) (Figure 5).

A time-course study was performed to examine the effects of different preincubation times on the magnitude of the inhibitory effect of dexamethasone pretreatment. Data for three individual cultures showed that dexamethasone (100 nM) when

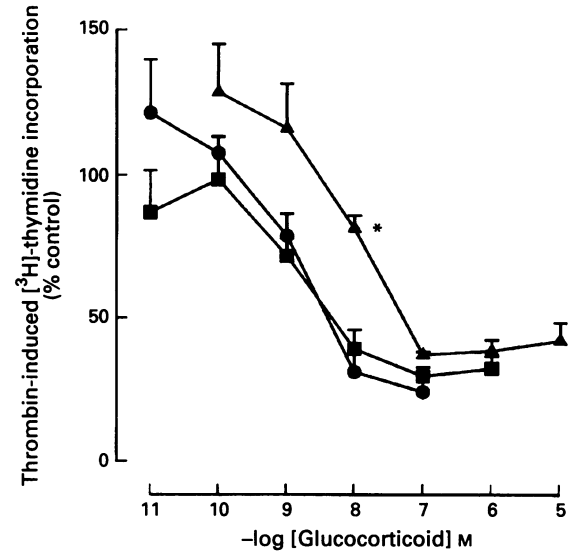

Figure 2 Concentration-response curves for inhibition by glucocorticoid pretreatment of thrombin (0.3 u ml^{-1})-stimulated [^{3}H]-thymidine incorporation. Results are expressed as a percentage of the control response to thrombin and represent the means and s.e.mean of at least 3 experiments in 3 different cultures, each experiment being carried out in quadruplicate: dexamethasone (■); methylprednisolone (●); hydrocortisone (▲). *$P<0.05$, ANOVA comparing curves in the presence of the different glucocorticoids.

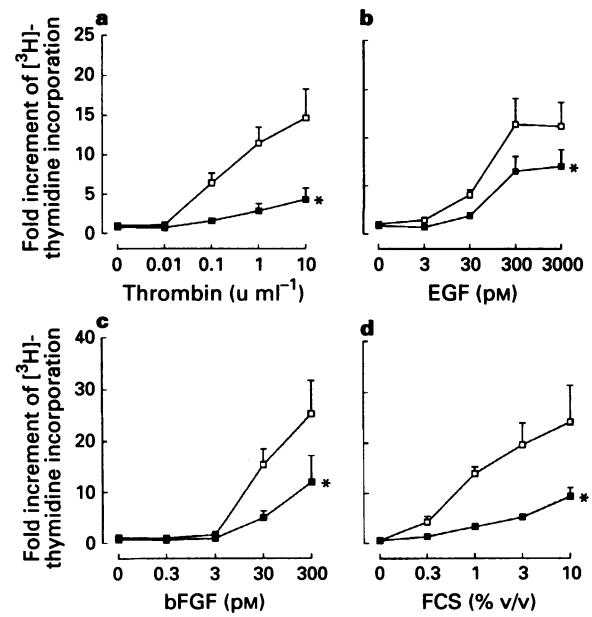

Figure 3 Effects of dexamethasone pretreatment on the incorporation of [^{3}H]-thymidine in response to (a) thrombin (0.01–10 u ml^{-1}), (b) EGF (3–3000 pM), (c) bFGF (0.3–300 pM) and (d) FCS (0.3–10% v/v). Results are expressed as the fold increment over the baseline incorporation of [^{3}H]-thymidine (in unstimulated cells) in the presence (■) and absence (□) of dexamethasone (100 nM) and are presented as means and s.e.means of at least 3 experiments in 3 different cultures, each experiment being carried out in quadruplicate. *$P<0.05$, ANOVA, comparing responses in the presence and absence of dexamethasone (100 nM). For abbreviations, see text.

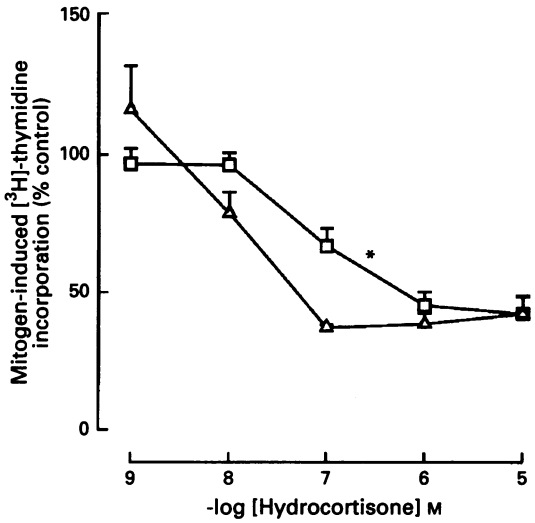

Figure 4 The effect of hydrocortisone (0.001 – 10 μM) on increases in [^{3}H]-thymidine incorporation stimulated by thrombin (0.3 u ml^{-1}, △) and by FCS (10%, □). Results are expressed as a percentage of the control response to the respective mitogen and represent the means and s.e.means of at least 3 experiments in 3 different cultures each experiment being carried out in quadruplicate. *$P<0.05$, ANOVA, comparing the concentration-response curves to hydrocortisone in the presence of 10% FCS with that in the presence of 0.3 u ml^{-1} thrombin.

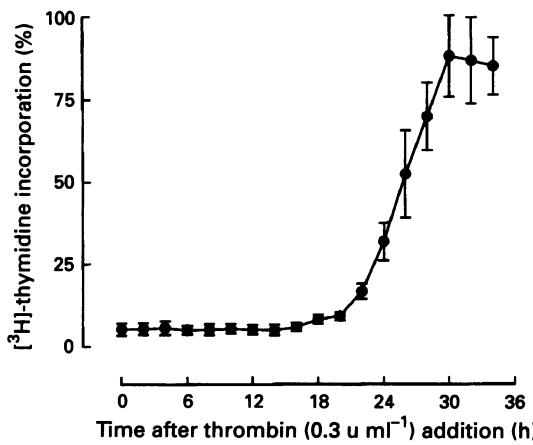

Figure 5 The time course of thrombin (0.3 u ml^{-1})-stimulated incorporation of [^{3}H]-thymidine. Cells were pulsed with 1 μCi ml^{-1} [^{3}H]-thymidine for 1 h at various times after thrombin addition up to 34 h. Individual results at the various time-points were expressed as percentages of the maximum [^{3}H]-thymidine incorporation for that individual culture. Data are presented as the mean ± s.e. of three experiments using three cell lines, each experiment being carried out in quadruplicate.

added up to 8 h before mitogen incubation caused a similar magnitude of inhibition to that when it was added 1 h before the mitogen (unpublished observations). In order to establish at which point in the cell cycle the inhibitory effect of dexamethasone was lost, a further time-course study was carried out by comparing the effects of dexamethasone pretreatment (1 h before thrombin) with those of post-treatment (1, 3, 6, 9, 11, 14, 17, 19, 21, 24 h) after thrombin addition. Maximum inhibition was observed at a 1 h pretreatment (response reduced to approximately 30% of control) (Figure 6). The inhibitory effect was observed when dexamethasone was added up to 17 – 19 h after thrombin stimulation, but not when added at 21 h or more post-thrombin.

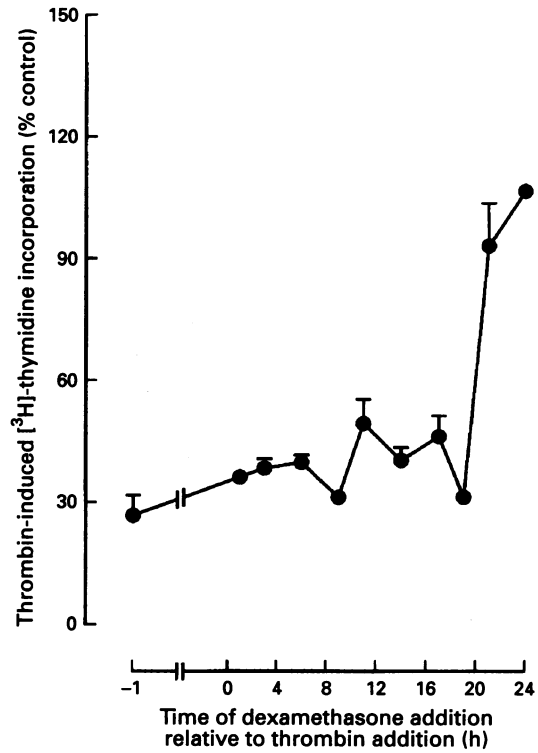

Figure 6 Effects of pre- and post-incubation with dexamethasone (100 nM) on the incorporation of [^{3}H]-thymidine. Dexamethasone was added at 1 h before and various times after thrombin and left in contact with the cells until the end of the experiment. Results are expressed as a percentage of the control response to thrombin (0.3 u ml^{-1}) and represent the means and s.e.mean of at least 3 experiments in 3 different cultures each carried out in quadruplicate (results for the timepoints 1, 9, 19, 24 h represent the averages of 2 experiments from 2 different cultures and hence no standard errors are shown).

Table 1 Effects of non-glucocorticoid steroids on thrombin (0.3 u ml^{-1})-stimulated increases in [^{3}H]-thymidine incorporation

	Fold increment in [^{3}H]-thymidine incorporation				
		concentration (nM)			
Steroid	Nil[1]	1	10	100	1000
Aldosterone	6.64 ± 2.45	6.17 ± 2.60	6.83 ± 2.64	5.80 ± 2.38	6.85 ± 2.47
Progesterone	8.87 ± 0.66	9.63 ± 1.00	9.97 ± 1.11	9.57 ± 1.02	9.43 ± 1.35
17β-Oestradiol	9.08 ± 1.42	9.19 ± 1.85	10.21 ± 2.00	9.46 ± 1.49	9.02 ± 1.29
Testosterone	10.71 ± 1.99	10.63 ± 1.79	9.73 ± 1.97	9.90 ± 1.82	10.00 ± 1.68

($n=3$ different cell lines from 3 different donors). [1] Nil represents the response to thrombin in the absence of steroid.

Discussion

The major finding of this study was that dexamethasone inhibited the proliferation of human cultured airway smooth muscle. This action is likely to represent a common effect of glucocorticoids, since hydrocortisone and methylprednisolone had similar inhibitory actions. In contrast, neither the mineralocorticoid, aldosterone nor the sex steroids, testosterone, progesterone or 17β-oestradiol had any inhibitory effect on thrombin-induced DNA synthesis at concentrations spanning the physiological levels of these steroids. Although glucocorticoids inhibited the responses to a range of mitogens, the magnitude of the inhibitory effect of dexamethasone was dependent on the mitogen, with responses to EGF being significantly less sensitive than those of the other mitogens.

Both thrombin and FCS elicited significant increases in cell number which were prevented by dexamethasone pretreatment. Thus, the inhibitory effect of dexamethasone on DNA synthesis is followed by a reduction in cell proliferation. There was no evidence of a cytotoxic effect of dexamethasone, since incubation for 48 h had no effect on resting cell number, protein synthesis or exclusion of trypan blue. Furthermore, under light microscopy there were no detectable changes in cell morphology in response to dexamethasone.

Potency comparisons between the glucocorticoids indicated a rank order of dexamethasone $\approx$ methylprednisolone > hydrocortisone. Other studies indicate that dexamethasone is between 5 and 10 times more potent than methylprednisolone (Zitnik *et al.*, 1994). There is no obvious explanation for the failure to detect a significant difference in the potency of dexamethasone and methylprednisolone in the present study. Hydrocortisone is structurally identical to cortisol, the physiological glucocorticoid in man. Interestingly, the concentration-range for hydrocortisone-mediated inhibition of the response to thrombin indicated a maximum inhibitory effect at 100 nM which is within the physiological range of cortisol levels (50–500 nM) reported in healthy subjects (Ditzel *et al.*, 1964) and in asthmatics (Nicolaizik *et al.*, 1994). In the cell culture medium used in these experiments there is no glucocorticoid and therefore the addition of 100 nM hydrocortisone may be considered as a physiological replacement, since airway smooth muscle cells *in situ* would be exposed to cortisol (hydrocortisone) levels of this order of magnitude. The large inhibitory effect of hydrocortisone at this concentration suggests that the airway smooth muscle proliferation component of the airway wall remodelling response in asthma may be regulated by endogenous steroids. However, extensive binding of endogenous steroids by corticosteroid binding globulin may reduce the free concentration of cortisol to 10% of the total (Wilckens, 1995). The question of whether supra-physiological concentrations of glucocorticoids would provide additional modulation of proliferation was assessed by using a more efficacious stimulus for mitogenic responses, namely a supramaximal concentration of FCS. Under these conditions, supraphysiological concentrations of hydrocortisone were required to produce maximal inhibitory effects, indicating that regulation of airway smooth muscle proliferation is a potential therapeutic action of inhaled and oral steroid treatment of asthma.

Dexamethasone (100 nM), at a maximum effective concentration for inhibition of responses to thrombin, also inhibited the [^{3}H]-thymidine incorporation stimulated by EGF, bFGF, and FCS. The EGF response seemed to be less sensitive to the inhibitory effect of dexamethasone than those to thrombin, bFGF, or FCS, raising the possibility that the components of the mitogenic signalling pathway affected by dexamethasone are of variable importance for these mitogens. In addition, this differential effect may provide some insight into the target for the inhibitory effect of the glucocorticoids.

Since glucocorticoids are used prophylactically in the treatment of asthma, the relationship between glucocorticoid exposure and that to mitogens is likely to be variable. Thus, time-course experiments were carried out examining both pre- and post-mitogen addition of dexamethasone. There was no difference in the magnitude of the inhibitory effect when dexamethasone was added up to 8 h before thrombin addition. Furthermore, the glucocorticoid was still an effective inhibitor of the thrombin response when added as late as 17–19 h after mitogen. Experiments using brief pulses with [^{3}H]-thymidine established that the duration of G_1 (the period between the quiescent G_0 and DNA synthesis S phases) was approximately 22 h. Thus, the observation of significant inhibition by dexamethasone when added up to 17–19 h after the addition of thrombin suggests that the target is not one of the well characterized initial steps in the signalling pathway for mitogenesis which include phospholipase C activation, transmembrane Ca^{2+} flux, protein kinase C activation and activation of receptor tyrosine kinases. It has recently been shown that thrombin (Tomlinson *et al.*, 1995) and other stimuli including histamine (Amrani *et al.*, 1994) induce only a transient (approximately 2 min duration) increase in intracellular calcium. Thus, despite evidence that thrombin increases intracellular calcium and data implicating these calcium increases in mitogenesis (Sung *et al.*, 1993; Baffy *et al.*, 1994), it is evident that addition of dexamethasone at 18 h after thrombin could not be acting by inhibition of the transient calcium increases induced by this mitogen. In contrast, signalling mechanisms which remain activated until the cells reach the restriction point (Sherr, 1994) in the G_1 phase of the cell-cycle, such as MAP kinase activation (Lenormand *et al.*, 1993), remain as possible targets for inhibition by glucocorticoids.

Amongst the potential mechanisms of action of the glucocorticoids in inhibiting cell proliferation is an action on the eicosanoid pathway, since recent studies indicate that a number of eicosanoids including thromboxane A_2 (Noveral *et al.*, 1992; Tomlinson *et al.*, 1994) and cysteinyl leukotrienes (Noveral & Grunstein, 1992) stimulate airway smooth muscle proliferation. Moreover, in vascular smooth muscle, inhibitors of eicosanoid production reduce proliferative responses to serum, bFGF and PDGF (Dethlefsen *et al.*, 1994). Alternative anti-mitogenic actions of dexamethasone include inhibition of the expression of immediate early genes such as *c-fos*, *c-myc*, and *c-jun* (Zhang *et al.*, 1991; Landers & Spelsburg, 1992). The immediate early response genes are known to be activated in response to thrombin and other mitogens in other cells types, including vascular smooth muscle and are considered to constitute early signals in biochemical pathways subserving mitogenesis (Mehmet & Rozengurt, 1991; Nishimura *et al.*, 1992; Obberghen-Schilling & Pouyssegur, 1993). In addition, the expression and production of autocrine growth factors such as platelet-derived growth factor AA and heparin-binding EGF in thrombin-stimulated vascular smooth muscle are inhibited by dexamethasone (Nakano *et al.*, 1993). The expression of these growth factors reach a peak 4–6 h after thrombin stimulation. However, the retention of the inhibitory effect of dexamethasone when added up to 17–19 h after thrombin, suggests that the target for this effect is either a later signal in the pathway for mitogenesis, or an early signal that is maintained at least until this time point and may be negated by the addition of the steroid immediately prior to the entry of the cells into S-phase.

The inhibitory effect of dexamethasone on proliferation of airway smooth muscle raises the possibility that one of its anti-asthma effects may be prevention of the airway wall thickening that occurs in asthma. Increases in airway wall thickness are likely to be a result of persistent inflammation in the airway wall. The clinical efficacy of glucocorticoid is greater if given early in the treatment regimen following diagnosis. There is a gradual improvement in airway function with chronic steroid treatment which may take as long as a year to reach a maximal therapeutic effect (Haahtela *et al.*, 1994). These clinical observations are consistent with actions on processes that are only slowly reversible, whereas the anti-inflammatory effects of glucocorticoids are evident following only two weeks of treatment (Kraan *et al.*, 1985). There are no data available on the reversibility of the airway wall remodelling in asthma but it seems reasonable to consider the possibility that reversal by

glucocorticoids of the airway wall remodelling response may account for the slow onset of maximal therapeutic effects of glucocorticoids.

In summary, dexamethasone has a differential inhibitory effect on the airway smooth muscle proliferation stimulated by a variety of mitogens. This inhibition represents a common effect of the glucocorticoids, but not of aldosterone or the sex steroids, and may involve an action on events late in G_1 and immediately before S-phase of the cell cycle. The potent inhibitory action of dexamethasone on airway smooth muscle proliferation may help explain the effectiveness of the glucocorticoids in the prophylactic, chronic treatment of asthma.

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