Induction of the E-selectin promoter by interleukin 1 and tumour necrosis factor α, and inhibition by glucocorticoids

Keith P. RAY*[1], Stuart FARROW*, Maria DALY*, Francois TALABOT† and Nicola SEARLE*

*Department of Cell Biology, Glaxo Wellcome Research and Development, Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, U.K., and †Geneva Biomedical Research Institute, Glaxo Wellcome Research and Development, S.A., 14 Chemain des Aulx, Case Postale 674, 128 Plan-les-Ouates, Geneva, Switzerland

Cytokine-induced expression of the endothelial cell surface adhesion molecule E-selectin is inhibited by glucocorticoids (GCs). To investigate possible mechanisms for steroid inhibition, a reporter gene (ESAP) was constructed, comprising the cytokine responsive region of the E-selectin gene (nt -383 to $+81$) coupled to alkaline phosphatase (AP). In A549 cells stably transfected with the ESAP gene, AP production was highly responsive to the cytokines interleukin 1β (IL-1β) and tumour necrosis factor α, with ED_{50} values of 3 pM and 1000 pM respectively. Furthermore the cytokine-induced AP responses were inhibited by GCs, indicating that both transcriptional activation and GC suppression of the E-selectin gene were mediated via regulatory elements within the same region of the promoter. The relative potencies of GC drugs as inhibitors of IL-1β (10 pM)-stimulated ESAP-gene activation were fluticasone > beclomethasone > dexamethasone, with IC_{50} values of 0.13, 1.1 and 2.7 nM respectively. Inhibition by fluticasone was blocked by the GC receptor (GR) antagonist drug mifepristone (Ru486), which is consistent with the suppressive effects of GCs being mediated via the GR. However, because the E-selectin promoter lacks a consensus glucocorticoid responsive element, mechanisms for inhibition independent of GR–DNA binding were investigated. Evidence that GCs also inhibited cytokine activation of a synthetic nuclear factor κB (NFκB)-driven reporter gene transiently transfected into A549 cells suggested that interference with the activation and/or function of this transcription factor was important for GC inhibition of ESAP. However, in A549-ESAP cells, fluticasone (100 nM) did not affect IL-1β (10 pM)-induced IkBα degradation, NFκB-p65 nuclear translocation or the DNA-binding capacity of nuclear NFκB complexes, over a period during which cytokine-induced ESAP-gene activation was inhibited. Finally, there was no evidence to suggest that GC enhancement of IkBα gene expression contributed to the suppression of the cytokine response. We conclude that interference by GR with the transcriptional activation potential of DNA-bound NFκB complexes might contribute to mechanisms underlying the anti-inflammatory effects of GCs.

INTRODUCTION

Glucocorticoids (GCs), which are used widely in the therapeutic control of acute and chronic inflammation, seem to exert their regulatory effects in different tissues and cells via the control of specific gene expression [1]. Depending on the particular gene involved, GCs can enhance or repress expression. GC enhancement is thought to be mediated via steroid binding and the activation of intracellular GC receptors (GRs). After translocation into the nucleus, the GR dimers can modulate transcription through binding to specific GC responsive elements (GREs) within the promoter regions of target genes. Regulation from the GRE within a particular promoter might also depend on GR interactions with other transcription factors. The mechanisms by which GCs exert their inhibitory effects on gene expression remain to be clearly established. GR binding to negative GREs has been proposed as one mechanism [2]. However, GRs suppress the activation of some genes via cytokine responsive promoter elements that lack GREs, including some proinflammatory cytokines [3–5], adhesion molecules [6,7], chemokines [8,9] and nitric oxide synthase [10]. This has suggested that mechanisms for transcriptional repression exist that are independent of GR–DNA binding. Furthermore, evidence that GC inhibition can occur at hormone concentrations lower than those required for GRE-dependent promoter activation, and that GR mutants lacking the ability to dimerize and bind GREs can still mediate repression, supports a model in which GR monomers are responsible for this function [11,12].

Possible mechanisms for GR-mediated gene suppression include interference with the activation, DNA binding or transactivation potential of other key transcription factors. Thus GCs inhibit AP-1 site-regulated gene expression via the direct GR binding of Fos–Jun heterodimers to interfere with their DNA-binding activity [13–15]. Interference with nuclear factor κB (NFκB) function might also represent a mechanism for GR-mediated repression of gene expression. The repressive effect of dexamethasone on tumour necrosis factor α (TNFα)-stimulated ICAM-1 gene expression in A549 cells has been localized to a region of the enhancer containing the ICAM–NFκB element [7]. NFκB also seems to be the target involved in GR-mediated repression of the IL8 [8] and CINC/gro [9] chemokine genes. Inhibition of IL6 gene expression involving NFκB association with either the GC or the oestrogen receptors has also been reported [3,5]. Possible mechanisms for these effects include GR interference with the processes involved in the activation and/or nuclear translocation of NFκB [8,9]. There is also evidence that a physical association between the GR and the p65 subunit of NFκB can directly inhibit NFκB–DNA binding [3,16] and might interfere with the synergistic cross-coupling of NFκB with other nuclear factors, including NF-IL6/CEBP-β, required for efficient gene activation [17,18]. Another mechanism that might contribute to the suppression of NFκB activity involves GC-mediated

Abbreviations used: AP, alkaline phosphatase; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility-shift assay; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; HUVEC, human umbilical-vein endothelial cells; IL-1β, interleukin 1β; NFκB, nuclear factor κB; TNFα, tumour necrosis factor α.

[1] To whom correspondence should be addressed.

transcriptional activation of the IkBα gene [19,20]. Because activation of the NFκB transcription factor system is utilized extensively in the regulation of genes expressed during immune, inflammatory and acute-phase responses, these effects of the GR might partly explain the wide anti-inflammatory actions of GCs.

An important part of the host response to tissue injury, infection or allergic stimulation involves the recruitment of leucocytes and some classes of lymphocytes from the blood [21]. E-Selectin is a member of the selectin family of endothelial cell-surface adhesion proteins and contributes to the binding and extravasation of neutrophils. The expression of E-selectin is specific to endothelial cells and is acutely up-regulated by pro-inflammatory cytokines including interleukin 1 (IL-1) and TNFα. Analysis of the E-selectin promoter has identified several regions involved in cytokine induction; several factors capable of interacting with these sites have been characterized [22]. Cytokine induction of the promoter seems to require co-operative interactions between sites containing NFκB, HMG-I(Y) and ATF [23,24] transcription factor complexes. The composition of an additional complex required for cytokine induction and termed NF-ELAM2 has yet to be established.

It has been reported that, in human umbilical-vein endothelial cells (HUVEC), the induction by endotoxin (lipopolysaccharide) of E-selectin mRNA accumulation and expression on the cell surface can be inhibited by GCs [25]. Because there is no evidence for the presence of GRE sites within the E-selectin promoter, the mechanism for this effect remains to be established, but interference with key transcription factors including NFκB maybe involved.In the present study we have investigated GC inhibitory regulation of the E-selectin promoter, and have been able to demonstrate that regions required for GC suppression are contained within the element identified previously as essential for cytokine induction of this gene [26,27]. Although cytokine induction of the E-selectin gene requires the activation of NFκB and binding of this transcription factor to sites within this same cytokine responsive element, we have been unable to confirm that GR interference with NFκB activation or DNA binding contribute to the inhibition of gene activation.

MATERIALS AND METHODS

Materials

Culture media, including Dubecco's modified Eagle's medium and RPMI 1640 medium, antibiotics and amino acid supplements, were from Flow Laboratories (Rickmansworth, Herts., U.K.). Foetal bovine serum was from Gibco BRL (Life Technologies, Paisley, Scotland), and sterile cell culture grade plasticware (Falcon) was from Beckton Dickinson. Human recombinant IL-1α (5×10^7 i.u./mg) and interleukin-1β (1.2×10^7 i.u./mg) were expressed in *Escherichia coli* and purified as described previously [28]. TNFα (human recombinant, expressed in yeast; 2×10^7 i.u./mg; 10 μg/ml), BSA, protease inhibitors antipain, leupeptin and pepstatin, and other chemical reagents including Hepes buffer, dithiothreitol and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Poly(dI-dC)·poly(dI-dC) for electrophoretic mobility-shift assay (EMSA) experiments was from Pharmacia Biotech (St. Albans, Herts, U.K.). [γ-^{32}P]ATP (approx. 3000 Ci/mmol) for the end-labelling of oligonucleotide probes, Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes for Western blotting, and reagents and film for ECL detection were from Amersham International (Little Chalfont, Bucks., U.K.). Precast polyacrylamide gels for electrophoresis of proteins were supplied by Novex (R&D Systems Europe, Abingdon, Oxon., U.K.). Polyclonal antisera for the immunodetection of NFκB subunits IkB-α (MAD-3), IkB-β and p65NFκB (RelA) were obtained from Santa Cruz Biotechnology (Autogen Bioclear UK, Devizes, Wilts., U.K.). The steroid compounds dexamethasone and hydrocortisone were from Sigma, and mifepristone (Ru486) was from Affinity Research Products (Exeter, Devon, U.K.). Fluticasone propionate (CCI18781), budesonide (GR160288X) and beclomethasone monopropionate (CCI2382) were prepared at GlaxoWellcome (Stevenage, Herts., U.K.).

Vector construction

A reporter gene system was constructed containing the cytokine responsive element of the E-selectin promoter coupled to a reporter gene, alkaline phosphatase (AP), which could be measured easily after cytokine stimulation of mammalian cells. Initially, the plasmid pSVneo was constructed by cloning the neomycin resistance gene into an *Eco*RI site of the vector pSV2, downstream of the SV40 promoter and upstream of two SV40 processing sequences (SV40.3′), which were separated by a linker containing unique cloning sites (pSVneo). The reporter gene contained a region of the E-selectin promoter, nt -383 to +81, shown previously to be sufficient to confer maximal cytokine induction [27], coupled to the gene encoding a secretable form of placental AP (sPAP). This construct was inserted into the *Eco*RI/*Xba*I site of the pSVneo linker to create the vector pSVneoELAMsPAP as follows (-SV40prom-*Hin*dIII-neo-SV40.3′-*Eco*RI-**ELAM-sPAP**-*Xba*I-SV40.3′-) (pESAPneo).

Transfection and culture of A549 cells

Human lung epithelial cells (A549 cell line) were cultured routinely in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with foetal bovine serum (10%, v/v), glutamine (2 mM) and antibiotics penicillin (100 i.u./ml) and streptomycin (100 μg/ml). Cells were passaged by trypsin treatment and dilution of the resulting cell suspension 1:10 every 3–4 days. For stable transfection, cells were seeded into 5 cm culture dishes (8×10^5 cells per dish) and cultured for 48 h. Cells were washed with warm Hepes-buffered saline (HBS) solution containing 50 mM Hepes, 140 mM NaCl, 0.7 mM Na_2HPO_4,$2H_2O$, 5 mM KCl and 5.6 mM glucose, adjusted to pH 7.1 at 22 °C. Transfections were performed with a standard protocol with calcium phosphate as described for adherent cells ([29], section 16.33). Plasmid DNA was purified with CsCl; transfections used 25 μg per plate. After transfection the cells were incubated in normal medium for 2 days, followed by selection in medium containing 1 mg/ml G418 (Geneticin, Gibco/BRL). After 2 weeks, individual colonies were isolated by ring cloning and then expanded. Several cell lines that responded to stimulation with IL-1β (10 pM) to produce placental AP were identified and one was selected for futher studies. These cells, termed A549-ESAP, were maintained routinely in culture medium containing 500 μg/ml G418. Transient transfection of A549 cells with reporter genes was performed by electroporation. Briefly, cells were trypsin-treated and resuspended in intracellular buffer [30] at a concentration of 2×10^7 cells/ml. Cells (150 μl) were incubated on ice in 0.4 cm cuvettes for 10 min with DNA plasmids (30 μg) encoding either ESAP or $(NF\kappa B)_4$TK–AP in a total volume of 200 μl. The reporter $(NF\kappa B)_4$TK–AP contained four tandem copies of the NFκB responsive element GGAAA-GTCCC coupled to a minimal HSVTk promoter upstream of the AP gene. Electroporation was performed with a Bio-Rad gene pulser set at 240 V and 960 μF. After electroporation, cells were rested for 10 min on ice and then diluted to 20 ml in complete culture medium and sampled into 96-well plates (200 μl per well). Cells were cultured overnight before assays were performed.

AP assay

To assess the effects of cytokines and steroids on the induction of the reporter gene system, A549-ESAP cells were seeded into microtitre plates (50000 cells per well) in normal medium (200 μl per well) and cultured overnight. Media were then removed and replaced with fresh medium containing cytokines (IL-1β or TNFα) with or without compounds. Compounds were dissolved in DMSO to give 1 mM stock solutions. Depending on the experiment, compounds and cytokines were diluted in medium to give twice the final concentration required and separate additions of 100 μl were made. Compounds were added to the cells approximately 5 min before addition of the cytokines unless otherwise described. After incubation of cells for the times indicated, media were transferred into a replica plate and heat-treated at 65 °C for 30 min to inactivate any endogenous AP derived from cells or serum. To determine secreted AP activity, an aliquot of medium (25 μl) was transferred from each well into a separate 96-well plate and 200 μl of assay buffer added containing 1 M diethanolamine/HCl, 0.28 M NaCl, 0.5 mM $MgCl_2$ and 5 mM *p*-nitrophenyl phosphate, pH 9.85 at 22 °C. To develop the reactions, plates were incubated at 37 °C for 60 min and the differences in attenuance at 405 nm and 650 nm read for each well with a 96-well plate reader (Molecular Devices). Under these conditions the AP reaction rates were linear for the period of development over a wide range of enzyme concentrations. For some assays the initial rates of AP activity were determined directly on the plate reader. Colour development during the assay was therefore proportional to the amount of AP enzyme secreted by cells during the period of experimental treatment. Normally, cell treatments were performed in triplicate and control incubations containing medium alone or medium plus solvent vehicle(s) were included on each plate to determine the background AP activity for unstimulated cells. The blank values were routinely subtracted from the experimental values to give the final values shown in the results section, in attenuance units.

Stimulation and preparation of cytoplasmic and nuclear extracts

To investigate the activation and localization of NFκB:IkB complexes after cytokine treatment of A549 cells, cytoplasmic and nuclear extracts were prepared for EMSAs and Western blotting analysis as follows. A549 cells were cultured to confluence in 80 cm^2 flasks and treated with cytokines with or without compounds as shown. At the end of the treatment period cell monolayers were washed three times with 10 ml of ice-cold PBS, and nuclear extracts for EMSA analysis were prepared essentially as described by Dignam et al. [31] Cells were scraped into 10 ml of buffer A containing 10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml antipain), 0.5 mM PMSF and 0.5 mM dithiothreitol, pH 7.9 at 4 °C, and centrifuged for 10 min at 1100 ***g***. Cell pellets were resuspended in 200 μl of buffer A containing 0.1 % Nonidet P40 and were left for 10 min on ice. The tubes were then vortex-mixed vigorously and microcentrifuged for 15 min at 10000 ***g***. The supernatants were retained and the pellets resuspended in extraction buffer B containing 420 mM NaCl, 20 mM Hepes, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25 % (v/v) glycerol, 0.5 mM dithiothreitol and protease inhibitors (5 μg/ml), adjusted to pH 7.9 at 4 °C. After 60 min the samples were vortex-mixed, centrifuged for 15 min at 10000 ***g*** and the supernatants were retained. Cell extracts were stored at −80 °C. Cell extracts for analysis by Western immunoblotting were prepared essentially as described above but including modifications to the buffers described elsewhere [32].

EMSA

To assess the presence of NFκB complexes in nuclear extracts prepared from cytokine-treated cells an EMSA was performed with a ^{32}P-labelled double-stranded oligonucleotide probe corresponding to the E-selectin promoter NFκB site 5′-GGATGCCATTGGGGATTTTCCTCTTTACTGG-3′ [27]. Incubations contained 3 μg of nuclear protein, 1 μg of poly[dI-dC], 1 μl of ^{32}P-labelled double-stranded ELAM oligonucleotide (approx. 10000 d.p.m.), 3 μl of assay buffer (5×) containing 50 mM Tris/HCl, 250 mM NaCl, 5 mM dithiothreitol, 5 mM EDTA, 25 % (v/v) glycerol, pH 7.5, and water to give a final volume of 15 μl. Samples were incubated at 22 °C for 90 min before loading on a non-denaturing polyacrylamide gel (6 %, w/v). After electrophoresis, the gel was transferred to a sheet of nitrocellulose (Hybond C extra; Amersham) and dried under vacuum. Radioactivity on the gel was detected by autoradiography.

Western immunoblotting

Cell cytoplasmic and nuclear extracts, prepared as described above, were subjected to SDS/PAGE on gradient (8–16 %, w/v) polyacrylamide gels (Novex) [33]. Separated proteins were transferred electrophoretically to nitrocellulose membranes and the blots were blocked by incubation in Blotto buffer [PBS containing 5 % (w/v) dried milk] overnight at 4 °C. Blots were incubated overnight at 4 °C with specific primary antibodies diluted in Blotto buffer, then washed three times in PBS containing 0.1 % Tween-20 before incubation with the appropriate second antibody–horseradish peroxidase conjugate for 2–4 h at room temperature. After further washing, specific antibody binding was detected by ECL with reagents supplied by Amersham and the results were recorded on photographic film.

Analysis of surface expression of E-selectin on cytokine-stimulated endothelial cells

HUVEC were prepared as described previously [27], seeded at 2×10^4 cells/cm^2 into six-well tissue culture plates and cultured to reach confluency. To assess the effects of steroids, compounds were added 1 h before the addition of TNFα (10 ng/ml) or IL-1α (100 i.u./ml). Cells were activated for the relevant periods then trypsin-treated briefly and resuspended in RPMI medium containing 10 % (v/v) foetal bovine serum and counted. Cells (5×10^5 per tube) were pelleted and resuspended in 100 μl of PBS containing 2.5 % (v/v) foetal bovine serum, 0.1 % sodium azide and the appropriate primary antibody (anti-E-selectin; British Biotechnology, Oxford, U.K.). The cells were left on ice for approx. 45 min to stain. Cells were then washed twice with PBS/FCS buffer (3 ml per wash) then stained with a fluorescein-labelled second antibody (1:30 dilution of Fab-specific anti-mouse FITC; Sigma). The cells were incubated on ice for 45 min then washed twice with PBS/FCS buffer before fixation and resuspension in 0.5 ml of ice-cold PBS containing 2 % (w/v) paraformaldehyde. The cells were stored overnight at 4 °C before flow-cytometric analysis of fluorescent cells was performed.

RESULTS

Effects of corticosteroids on cytokine-induced E-selectin expression on HUVEC

The adhesion molecule E-selectin is expressed transiently on the surface of cytokine-induced endothelial cells, reaching a maxi-

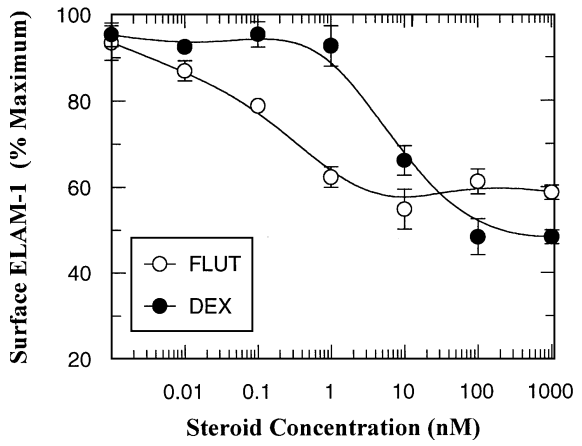

Figure 1 Effects of GCs on TNFα-induced E-selectin expression on HUVEC

HUVEC were stimulated with TNFα (10 ng/ml) in the absence or presence of increasing concentrations of dexamethasone (●) or fluticasone propionate (○). After 4 h, cell incubations were terminated and cell surface levels of E-selectin were measured by immunofluorescent staining and flow cytometry as described in the Materials and methods section. For each steroid concentration the percentage inhibition of E-selectin expression is given compared with the level measured on the cytokine-treated control cells. Each point represents the mean ± S.D. for three separate replicate incubations and the results are representative of those achieved in several experiments.

mum level at approx. 4 h after the onset of stimulation [27]. To determine the effects of GCs on this response, HUVEC were treated with steroids for 5 min before the addition of TNFα, and E-selectin levels were investigated by fluorescence-activated cell sorting analysis at 4 h. As shown in Figure 1, TNFα at 10 ng/ml strongly induced E-selectin expression and this response was inhibited (by 50–60 %), but not completely prevented, by GCs. For inhibition by dexamethasone and fluticasone, the approximate IC_{50} values were 10 and 0.1 nM respectively. Time course experiments revealed that the steroids were most effective when included before TNFα and that the ability to cause inhibition of the cytokine effect was lost completely if steroid addition was delayed beyond the 1–2 h post-induction period (results not shown).

E-selectin promoter reporter system

Previous studies of E-selectin gene regulation [26,27] identified *cis*-acting transcriptional control regions responsible for the cytokine-induced expression. To facilitate further studies of the mechanisms involved in cytokine induction and steroid repression of this gene, a fragment of the E-selectin promoter including the transcriptional start site and a 5′ flanking region containing the cytokine responsive element (−383 to +81) was used to create a promoter–reporter construct. This construct, termed pESAPneo, included a reporter gene encoding a secretable form of the heat-stable placental AP. After transfection of A549 cells with this construct followed by selection of G418-resistant clones, a stably transformed line was isolated that could be induced to secrete AP by cytokine stimulation. As shown in Figure 2, these cells showed no basal production of AP under normal growth conditions, but after the addition of either IL-1β or TNFα, release of AP from the cells could be detected after a delay of approx. 5 h. There was no detectable increase in the cellular content of AP during this lag period, suggesting that the AP was not accumulated before secretion. For cells incubated in the presence of IL-1β, there was an initial lag period before AP could

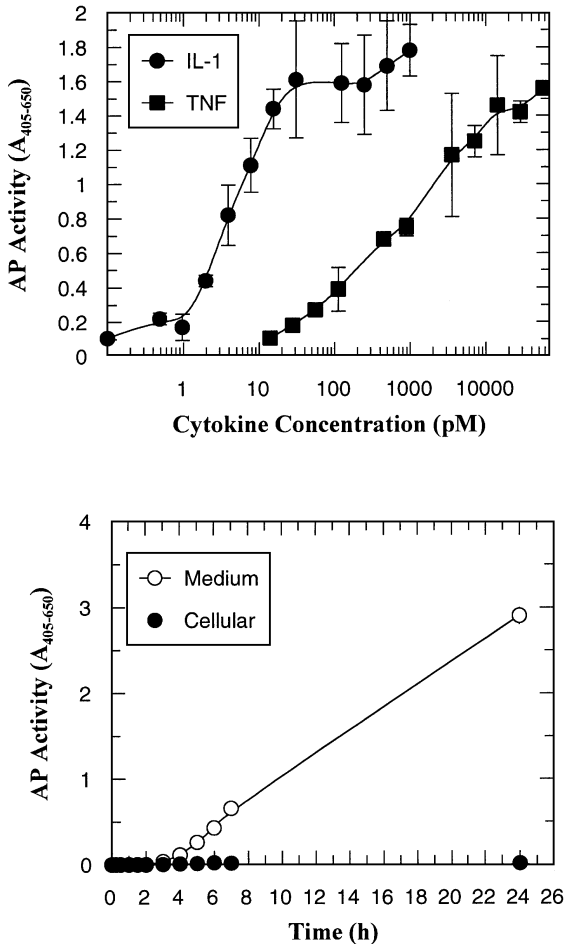

Figure 2 Cytokine induction of the E-selectin promoter–AP-reporter gene in A549 cells

A549-ESAP cells were stimulated overnight with various concentrations of IL-1β (0.6–1000 pM) or TNF-α (0.06–10 ng/ml) (upper panel), or for various times with IL-1β (10 pM) (lower panel). At the appropriate times media were removed and AP was measured. Samples from IL-1β-stimulated (●) and TNF-stimulated (○) cells (upper panel) and in media (○) and cell extracts (●) from IL-1β (10 pM)-treated cells (lower panel) were treated as described in the Materials and methods section. Colour development in the AP assay was measured as the attenuance (405–650 nm) after 1 h. Each point represents the level of AP activity (mean ± S.D.) obtained from three replicate samples.

be detected but then the rate of AP accumulation was approximately linear over a period of 24 h (Figure 2, lower panel). The level of production during this period was related to the concentration of IL-1β (Figure 2, upper panel), with half-maximal stimulation of the response at 3 pM and a maximal effect at 10 pM. TNFα was much less potent, with half-maximal stimulation occurring at approx. 1000 pM. Results confirmed that the selected region of the E-selectin promoter was responsive to cytokines when transfected into the A549 cells.

Effects of GCs on E-selectin promoter–AP reporter

Because this fragment of the E-selectin promoter retained elements required for cytokine responsiveness but lacked a consensus GRE we were interested to determine whether activation of the reporter gene would be sensitive to GC inhibition as seen with E-selectin (Figure 1). To investigate the effects of GCs on induction of the E-selectin promoter, A549-ESAP cells

Table 1 Steroid inhibition of IL1-induced AP reporter gene expression in A549 cells

A549 cells stably transfected with an E-selectin promoter reporter gene (AP) construct were stimulated with IL-1β (10 pM) in the absence or presence of increasing concentrations of different steroid drugs, as described in the Materials and methods section. After 24 h the media were removed for the determination of secreted AP and dose–response curves for steroid inhibition of the cytokine effect were plotted. IC_{50} values were determined with the Grafit curve-fitting and statistical analysis package. Individual values are means $\pm$ S.E.M. for *n* different experiments.

Steroid	IC_{50} (nM)	Inhibition (% of maximum)	*n*
Fluticasone	0.128 $\pm$ 0.04	65.3 $\pm$ 4.2	8
Dexamethasone	2.65 $\pm$ 0.91	69.2 $\pm$ 8.0	4
Budesonide	1.26 $\pm$ 0.68	69.2 $\pm$ 6.7	4
Beclomethasone	1.05 $\pm$ 0.13	68.0 $\pm$ 10.3	4
Hydrocortisone	140	–	1
Corticosterone	> 5000	–	1

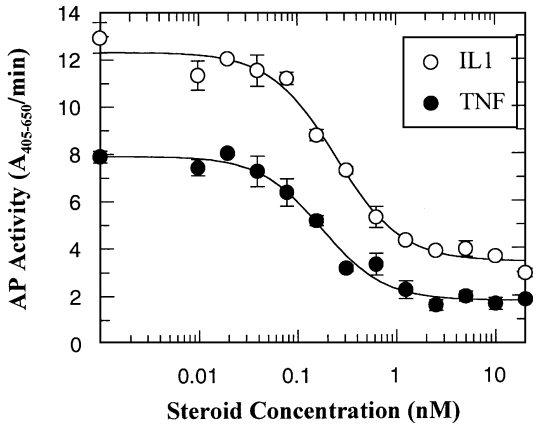

Figure 3 Fluticasone inhibits cytokine stimulation of an E-selectin promoter-coupled AP reporter gene

A549 cells stably transfected with an E-selectin promoter-coupled AP reporter gene were stimulated with IL-1β (10 pM, ○) or TNFα (10 ng/ml, ●) in the absence or presence of increasing concentrations of fluticasone, as described in the Materials and methods section. After incubation overnight (18 h), media were removed and tested for heat-stable AP activity. Individual points represent the mean $\pm$ S.D. for triplicate wells; results of a representative experiment are presented.

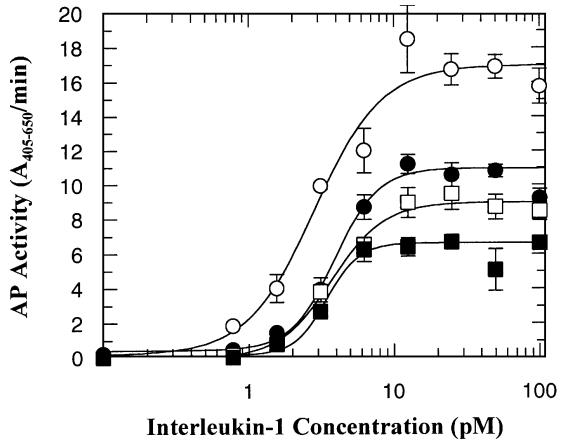

Figure 4 Effects of fluticasone on IL-1β-induced activation of an E-selectin promoter-coupled reporter gene

A549-ESAP cells were stimulated with a range of concentrations of IL-1β in the absence (○) or presence of 0.1 nM (●), 1 nM (□) or 10 nM (■) fluticasone. After incubation for 18 h, media were removed for the assessment of AP production as described in the Materials and methods section. AP activity was measured in three replicate wells and for each treatment the mean $\pm$ S.D. is given. Results shown are representative of those from three separate experiments.

were incubated overnight with IL-1β or TNFα in the presence or absence of a variety of different steroids. Results presented in Table 1 show that dexamethasone and related GCs have significant inhibitory activity with an order of potency fluticasone > dexamethasone > hydrocortisone, and IC_{50} values of 0.13, 2.7 and 140 nM respectively. As shown in Figure 3, fluticasone produced dose-related inhibitory effects on both IL-1-stimulated and TNFα-stimulated AP accumulation, with half-maximal effects at 0.24 and 0.17 nM respectively, in this experiment. Fluticasone did not cause complete inhibition of either the IL-1-stimulated or TNFα-stimulated responses and at maximally effective steroid concentrations (10–100 nM) only 60–70 % of the cytokine response was blocked (Table 1). The degree of inhibition was not significantly increased by extending the period of fluticasone pretreatment up to 1 h before cytokine addition, nor did we find other GCs to be any more effective (Table 1).

To assess whether GC inhibition might be mediated via a decreased sensitivity of the cells to IL-1 stimulation, rather than by interference with downstream regulation of gene expression, dose–response curves for IL-1-induced AP production were measured at various concentrations of fluticasone. Results showed that at each concentration tested, fluticasone acted to suppress the maximal response to IL-1 (Figure 4) rather than to decrease the sensitivity of the cells to IL-1 stimulation. These results therefore suggested that both cytokine induction and GC inhibition were mediated via the E-selectin promoter elements, which had been incorporated into the reporter gene.

Experiments with Ru486, a GR antagonist, demonstrated that this compound lacked inhibitory activity on its own but could completely reverse the inhibitory effects of fluticasone on IL-1-induced AP production (Figure 5b). Furthermore at increasing concentrations of Ru486 the dose–response curve for fluticasone inhibition was shifted to increasing concentrations (Figure 5a), indicative of the drug's ability to act competitively, and consistent with a model of GC suppression mediated via the GR.

In the earlier time course experiments we had observed that in IL-1-treated cells AP production was detected only 4–5 h after the onset of cytokine stimulation, and then remained linear for up to 24 h. Because the activation of factors required for transcriptional induction (including NFκB) in the A549 cells was known to be rapid (0–1 h), we were interested to determine whether the GC inhibitory effect was limited to this early 'inductive' phase or could occur after the onset of AP production had been established. As shown in Figure 6(a), the addition of a maximally effective inhibitory concentration of fluticasone (100 nM), before the onset of IL-1β (10 pM) stimulation, suppressed the subsequent rate of of AP accumulation. However, when added 24 h after the onset of IL-1 stimulation, fluticasone had no significant effect on AP production. A more detailed study showed that the capacity of the steroid to inhibit the cytokine response was gradually lost over a period of 2–3 hours (Table 2). Taken together, these results suggest that the mechanism for steroid inhibition was likely to involve interference with the initial stage of cytokine-induced E-selectin promoter

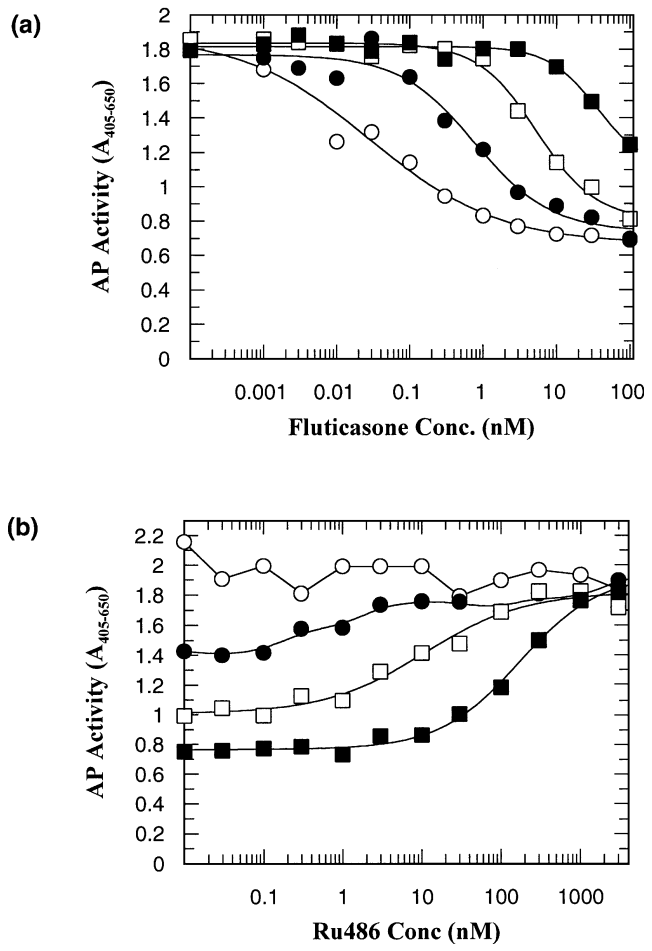

Figure 5 Antagonism of fluticasone inhibition by Ru486

A549-ESAP cells were treated with IL-1β (10 pM) and increasing concentrations of fluticasone (0–100 nM) in the absence (○) or presence of Ru486 at concentrations of 10 nM (●), 100 nM (□) or 1000 nM (■) (**a**). Alternatively, cells were treated with IL-1β (10 pM) and increasing concentrations of Ru486 in the absence (○) or presence of 0.1 nM (●), 1 nM (□) or 10 nM (■) fluticasone (**b**). After incubation overnight (18 h), cell media were removed for the assessment of AP production as described in the Materials and methods section. Each point represents the mean ± S.D. for three replicate wells. Similar results were obtained in three separate experiments.

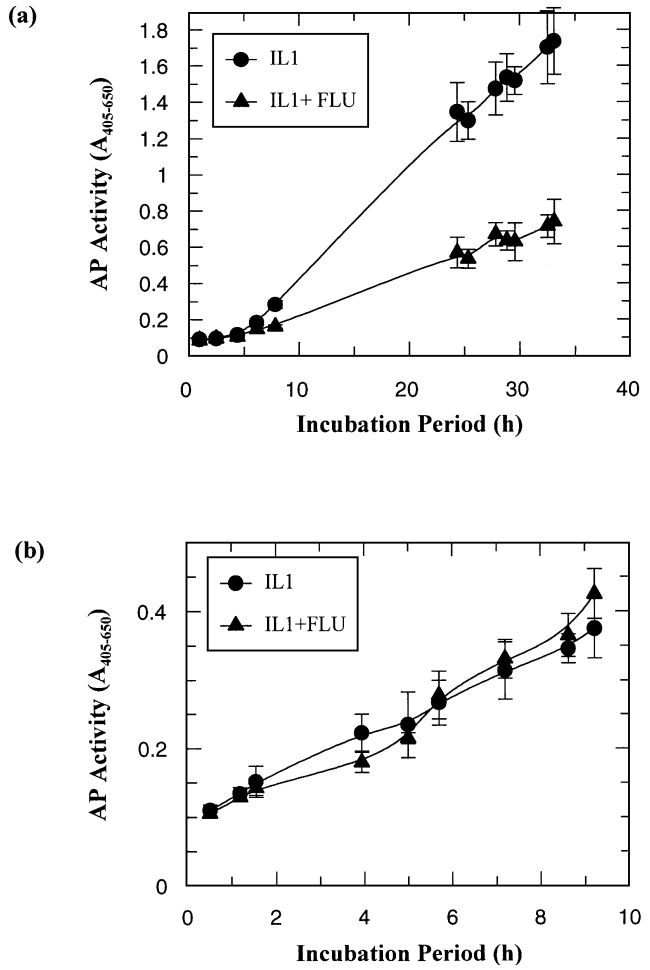

Figure 6 Effect of time of fluticasone addition on inhibition of IL-1-induced ESAP-reporter gene expression

The ability of of fluticasone to inhibit cytokine induction of ESAP in untreated and IL-1-prestimulated cells was assessed. A549-ESAP cells were incubated with IL-1β (10 pM) in the absence (●) or presence (▲) of fluticasone (100 nM), and AP production was assessed at the times shown. Fluticasone was added either immediately before the addition of IL-1β (10 pM) (**a**) or 24 h after the cytokine treatment had been started (**b**). For the cytokine-pretreated cells (**b**), media were replaced at 24 h with medium containing fresh IL-1β (10 pM) with fluticasone (100 pM). Samples were removed at the times indicated and AP activities were measured as described in the Materials and methods section.

activation rather than effects on mRNA stability or inhibition of protein synthesis.

Requirement of NFκB sites for fluticasone inhibition

Studies on the E-selectin promoter have shown that sites binding both NFκB and AP-1-like complexes are required for full cytokine induction [27]. To determine whether interference with NFκB function was a contributory factor in the mechanism for GC suppression, effects of GCs were reinvestigated with a reporter containing only NFκB responsive elements. A549 cells were transfected transiently with plasmids containing either the E-selectin promoter–reporter ESAP or a synthetic NFκB responsive reporter (NFκB-Tk-AP). Basal activity of both reporter genes was evident in the transiently transfected cells (Table 3); nevertheless IL-1 stimulated levels 3–4-fold and the induction of both reporter genes was inhibited by fluticasone. These results suggest that NFκB-binding sites within the E-selectin promoter are involved in mediating the GC repression of cytokine-induced gene expression.

Effects of fluticasone on NFκB activation

Previous studies have shown that NFκB is required for cytokine induction of the E-selectin promoter [27] and that this transcription factor is rapidly activated in response to IL-1 stimulation of A549 cells [33]. We therefore investigated whether the inhibitory effect of GCs might involve interference with NFκB activation, nuclear translocation or DNA binding. For these studies A549 cells were treated with IL-1β (10 pM) with or without fluticasone (100 nM), and properties of the cytoplasmic and nuclear forms of NFκB were investigated by immunoblotting and EMSA analysis. Cytokine treatment led to a rapid but

Table 2 Effect of time of fluticasone addition on inhibition of IL-1β-induced ESAP-reporter gene expression

A549-ESAP cell incubations were initiated by the addition of IL-1β (10 pM), fluticasone (100 nM) or control medium. Fluticasone was added simultaneously with IL-1β or at the times shown after the addition of IL-1β and incubations continued for a period of 18 h. Media were then removed and AP production was assessed as described in the Materials and methods section. Each value of AP activity is the mean $\pm$ S.D. for three replicate samples, and results from a representative experiment are shown.

Period before addition of fluticasone (h)	AP activity ($A_{405-650}$)
0	0.47 $\pm$ 0.03
0.5	0.64 $\pm$ 0.04
1.0	0.81 $\pm$ 0.06
2.0	1.08 $\pm$ 0.08
3.7	1.40 $\pm$ 0.10
6.0	1.46 $\pm$ 0.10
8.0	1.49 $\pm$ 0.09
Control (8.0)	1.49 $\pm$ 0.07

Table 3 Effects of IL-1 and fluticasone on NFκB-dependent reporter gene expression in transiently transfected A549 cells

A549 cells were transfected by electroporation with E-selectin promoter (ES-NFκB) and $(NF\kappa B)_3$-TK promoter (TK-NFκB) reporter gene expression plasmids, as described in the Materials and methods section. After 24 h, cells were treated overnight with control medium or IL-1β (10 pM) in the absence or presence of fluticasone (100 nM). AP production was measured in cell supernatants as described. Values are means $\pm$ S.D. for four replicate samples; results of a representative experiment are shown.

	AP activity ($A_{405-650}$)	
Additions	TK-NFκB	ES-NFκB
None (control)	0.36 $\pm$ 0.06	0.22 $\pm$ 0.03
IL-1β	1.55 $\pm$ 0.09	1.23 $\pm$ 0.04
IL-1β + fluticasone	0.82 $\pm$ 0.06	0.81 $\pm$ 0.12

transient loss of cytoplasmic IkBα (Figure 7a, upper panel). After the addition of cytokine there was a substantial loss, within 30 min, of the IkBα detectable in cytoplasmic extracts by Western blotting. Levels of IkBα were restored after 2 h and remained constant over the subsequent 24 h period. In addition to the expected major band of IkBα immunoreactivity (approx. 40 kDa) we detected a second band (approx. 24 kDa), which was strongly induced after stimulation with IL-1β (Figure 7a, upper panel). The nature of this band is not known but its cross-reactivity suggests that it might be derived from IkBα or represent a closely related gene product. The presence of fluticasone had no obvious effect either on the loss of IkBα seen after 30 min or on the subsequent reappearance of this protein. There was no detectable effect of IL-1 or fluticasone on the levels of IkBβ (results not shown). The changes in IkBα levels measured in cytoplasmic cell extracts were well correlated with the appearance in nuclear extracts of the NFκB-p65 subunit (Figure 7a, lower panel). Thus nuclear NFκB-p65 protein was almost undetectable on Western blots with extracts prepared from unstimulated cells but after IL-1 stimulation there was a strong induction within 30 min. Subsequently, levels of NFκB-p65 fell back and by 24 h were the same as those detectable in nuclear extracts prepared from unstimulated control cells. Comparison with the extracts prepared from fluticasone-treated cells showed that the steroid had no effect on the nuclear changes in NFκB p65 compared with the controls. Nevertheless measurements of AP production in the same experiment (Figure 7b) showed that addition of the steroid had produced an inhibition.

These results clearly demonstrate that the steroid did not interfere with the process of NFκB activation involving IkB degradation or the nuclear translocation of complexes containing the NFκB-p65 subunit. To establish whether GR interactions might affect a form of NFκB binding specifically to the E-selectin DNA-binding site, gel-retardation experiments were performed with a ^{32}P-labelled probe based on the E-selectin gene promoter NFκB site [27]. In unstimulated cells no retardation of the probe was detected (Figure 8); however, after stimulation of cells with IL-1 for 1 h a highly induced complex was detected. Previous studies have confirmed this complex to be NFκB [34]; its formation can be inhibited with antibodies to NFκB-p50/p65 (results not shown). There was no effect of either fluticasone or dexamethasone on the cytokine-induced formation of this complex, suggesting that the steroid inhibitory effect is not mediated

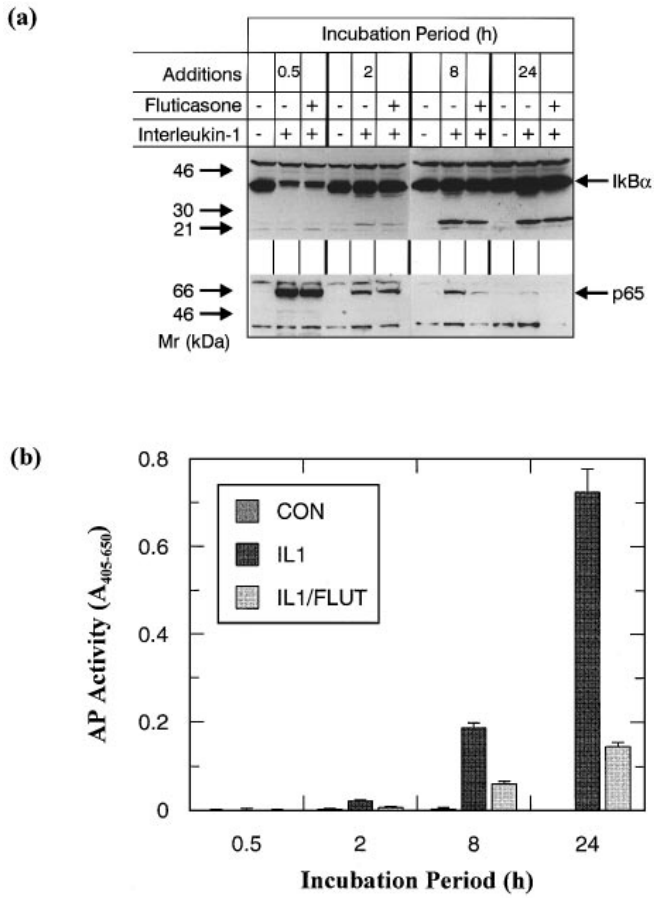

Figure 7 Effects of fluticasone on IL-1-induced IkB degradation, NFκB-P65 nuclear translocation and ESAP gene activation in A549 cells

(**a**) A549-ESAP cells (one 75 cm^2 flask per point) were incubated with medium containing additions as shown. At the times indicated (0.5–24 h), cells were harvested and cytoplasmic and nuclear extracts were prepared as described in the Materials and methods section. Equal amounts of protein extract were loaded [approx. 65 μg of cytoplasmic extract (upper panel); 80 μg of nuclear extract (lower panel)] and samples were subjected to SDS/PAGE. Western immunoblotting was performed with IkBα-specific (upper panel) and NFκB-P65-specific (lower panel) antibodies, with ECL detection. Positions of molecular mass standards (in kDa) are shown at the left; arrows at the right indicate the expected positions of IkBα and P65 proteins. (**b**) AP activity was measured in culture media removed from the cells incubated as described for (**a**), by following the procedure described in the Materials and methods section.

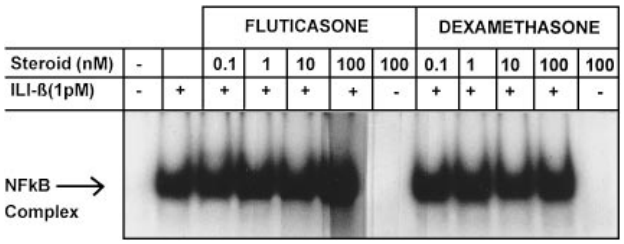

Figure 8 Effects of GCs on IL-1 induction of nuclear NFκB in A549-ESAP cells

A549-ESAP cells were incubated with or without IL-1β (1 pM) in the presence or absence of fluticasone (0.1–100 nM) or dexamethasone (0.1–100 nM). After 60 min, incubations were terminated and nuclear extracts were prepared as described in the Materials and methods section. An EMSA was performed with a ^{32}P-labelled probe based on the E-selectin NFκB site as described; complexes retarded on the 5% (w/v) polyacrylamide gel were detected by autoradiography.

by interference of the GR with the nuclear activation or DNA binding of a promoter-specific form of NFκB.

DISCUSSION

The cytokine-induced up-regulation of E-selectin expression on vascular endothelial cells is thought to play an important part in the recruitment of leucocytes from the blood into sites of tissue inflammation. Recent studies have demonstrated that the mechanisms governing the control of E-selectin gene transcription are particularly important in this process, and key regulatory elements of the E-selectin gene promoter have been mapped [26,27]. Previous work [25] and our own results (Figure 1) have demonstrated that GCs can have a potent inhibitory effect on the induction of E-selectin expression in endothelial cells: evidence in this study suggests that at least part of this effect is mediated via interference with events regulating transcriptional activation via the E-selectin gene promoter.

To investigate possible mechanisms involved in the GC inhibition of E-selectin gene expression, we made use of a fragment of the E-selectin gene promoter that had been reported to contain the cytokine responsive regulatory elements [27]. Our results confirmed that when this element was fused upstream of a reporter gene (AP) in stably transfected human lung epithelial A549 cells, expression of this enzyme was highly inducible with the cytokines IL-1 and TNFα. The kinetics of cytokine-induced AP production in these A549 cells differed from the rapid but transient induction of E-selectin expression in HUVEC [27]. In particular, the onset of AP production after stimulation with IL-1 was delayed for approx. 4 h but secretion then continued at an elevated rate for up to 24 h. These differences might have arisen from the use of only part of the natural promoter region in the reporter construct or they might reflect differences in the cellular context or balance of regulatory factors present in epithelial compared with endothelial cells.

Despite these differences, the sensitivity of the cytokine induction process to GC inhibition was still evident when using the ESAP reporter system. Thus our results indicate that the mechanisms for both induction and repression of the E-selectin gene involve control elements located within the same region of the enhancer. Evidence that dexamethasone and fluticasone, drugs with known GR agonist activity, caused inhibition in the A549 cell assay and that this effect could be reversed by the selective GR antagonist Ru486 suggest that the inhibitory effects of these steroids are mediated via the GR. Evidence that fluticasone did not decrease the sensitivity of A549-ESAP cells to IL-1 stimulation argues against an effect of GCs on receptor number or affinity. Rather, results showing that GCs inhibited the maximal responses to both IL-1 and TNFα were consistent with suppression's being exerted on a common regulatory event downstream of cytokine receptor activation. However, we have noted that even at their maximally effective concentrations, none of the steroids tested caused the complete suppression of either cytokine-induced AP production (A549) or E-selectin expression (HUVEC). Possible mechanisms for the GR-mediated inhibitory effects include interference with cytokine signalling, translocation of cytoplasmic transcription factors, transcriptional activation, mRNA stability or protein synthesis. Results showing that a part of the E-selectin promoter region required for cytokine induction could also confer susceptibility to steroid inhibition suggested a mechanism involving GR interference with the cytokine induction process. Furthermore time course studies showing that steroid inhibition was maximal only when the steroid was included during the initial induction period of cytokine stimulation are consistent with interference's being exerted at the level of the cytokine-regulated transcriptional activation rather than via later events governing protein expression.

These studies together indicated that GC/GR suppression was mediated via the cytokine responsive region of the E-selectin promoter. However, the absence of specific GC regulatory elements within this region of the promoter pointed to a mechanism of negative regulation independent of GR–DNA binding. Several recent reports have shown that GR-mediated interference with the nuclear localization or DNA-binding properties of other key transcription factors can provide alternative mechanisms for the inhibition of regulated gene expression. Within the E-selectin promoter, several regulatory sites seem to contribute to the overall cytokine induction [22]. In particular, the occupation of NFκB sites by complexes containing NFκB p50 and p65 subunits has been described after the stimulation of cells with IL-1/TNF; these sites seem to be essential for the cytokine activation of transcription. We have shown previously [34] and in the present study that both NFκB and the NFκB-dependent E-selectin promoter can be activated by cytokines in A549 cells. We have also shown that GCs can inhibit cytokine activation of both E-selectin and synthetic NFκB-dependent promoter–reporter genes, suggesting that NFκB is an important target for GR-mediated transcriptional repression. Interference with the cytokine-induced nuclear localization or DNA-binding properties of this transcription factor has been implicated recently in the GR suppression of several cytokine-inducible genes including ICAM-1, IL6 and IL8 [3,4,6–8]. However, studies in the A549 cell system have not confirmed that either of these mechanisms is important for suppression of the E-selectin promoter. Thus GCs had no apparent effect on the IL-1-induced disappearance of IkBα, the nuclear translocation of NFκB or the ability of nuclear NFκB complexes to bind to a DNA probe. It remains a possibility that, whereas the GR does not interfere with these processes, it might interfere with interactions between NFκB and other components, either within NFκB sites or at other sites regulating transcription. For example, recent studies suggest that GRs might compete with other transcription factors for binding to a limited pool of co-activator molecules such as P300/CBP, which mediate interactions with the basal transcription machinery [35]. Evidence that both nuclear steroid receptors [35] and RelA(p65) [36] can bind to N-terminal regions of p300/CBP is therefore suggestive of a mechanism for GR inhibition involving competition for P300/NFκB interactions.

An alternative mechanism proposed recently has suggested that GRs might influence NFκB-dependent gene activation

through the induction of IkBα gene expression, to inhibit the initial activation or cause more rapid sequestration and inactivation of activated nuclear NFκB complexes. However, again our results do not support such a mechanism in A549 cells. We found that GC treatment had no apparent effect on the initial cytokine-induced loss or subsequent reappearance of IkBα (Figure 7) compared with the levels observed in cytoplasmic extracts from cells treated with IL-1 alone. In addition there was no apparent effect of GCs on the nuclear levels of p65 over the initial period during which GR-mediated inhibition was exerted. Interestingly, in the cytokine-stimulated A549 cells results showing that IkBα levels were restored to normal by the time that AP production was greatest suggest that NFκB activity is required only during the initial period of transcriptional activation. It seems likely that the sustained output of AP observed after cytokine induction reflects a high degree of AP mRNA stability rather than a sustained increase in AP mRNA transcription. However, this evidence again points to GR-mediated inhibition's being exerted during the early phase of cytokine-induced transcriptional activity. We conclude that steroid inhibition of cytokine-induced E-selectin expression on endothelial cells involves GR-mediated interference with the transcriptional activation process. A mechanism involving GR interference of interactions between NFκB and other components of the transcriptional activation complex is suggested.

After submission of this manuscript Brostjan et al. [37] reported similar results, showing that in pig endothelial cells lipopolysaccharide- or TNFα-induced E-selectin expression is inhibited by glucocortiocoids via a mechanism involving interference with transcriptional activation. Interestingly, their results also suggest that GC repression is mediated via NFκB rather than NF-ELAM1 sites within the E-selectin promoter. Furthermore, evidence that dexamethasone did not change cytokine-induced NFκB DNA-binding activity is in agreement with results presented here and points to a mechanism of GC repression involving NFκB trans-activation as proposed above.

We thank John Delamarter for the gift of the E-selectin promoter reporter gene, and Roberto Solari for helpful discussions and a critical review of this manuscript.

REFERENCES

1 Cupps, T. R. and Fauci, A. S. (1982) Immunol. Rev. **65**, 133–155
2 Sakai, D. D., Helms, S., Carlstedt-Duke, J., Gustafsson, J. A., Rottmen, F. M. and Yamamoto, K. R. (1988) Genes Dev. **2**, 1144–1154
3 Ray, A. and Prefontaine, K. E. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 752–756
4 Stein, B. and Young, M. X. (1995) Mol. Cell. Biol. **15**, 4971–4979
5 Ray, A., Prefontaine, K. E. and Ray, P. (1994) J. Biol. Chem. **269**, 12940–12946
6 Van de Stolpe, A., Caldenhoven, E., Raajmakers, J. A. M., Van der Saag, P. T. and Koenderman, L. (1993) Am. J. Respir. Cell Mol. Biol. **8**, 340–347
7 Van de Stolpe, A., Caldenhoven, E., Stade, B. G., Koenderman, L., Raaijmakers, J. A. M., Johnson, J. P. and van der Saag, P. T. (1994) J. Biol. Chem. **269**, 6185–6192
8 Mukaida, N., Morita, M., Ishikawa, Y., Rice, N., Okamoto, S., Kasahara, T. and Matsushima, K. (1994) J. Biol. Chem. **269**, 13289–13295
9 Ohtsuka, T., Kubota, A., Hirano, T., Watanabe, K., Yoshida, H., Tsurufuji, M., Iizuka, Y., Konishi, K. and Tsurufuji, S. (1996) J. Biol. Chem. **271**, 1651–1659
10 Kleinert, H., Euchenhofer, C., Ihrig-Biedert, I. and Forstermann, U. (1996) Mol. Pharmacol. **49**, 15–21
11 Jonat, C., Rahmsdorf, H. J., Park, K.-K, Cato, A. C. B., Gebel, S., Ponta, H. and Herrlich, P. (1990) Cell **62**, 1189–1204
12 Heck, S., Kullmann, M., Gast, A., Ponta, H., Rahmsdorf, H. J., Herrlich, P. and Cato, A. C. B. (1994) EMBO J. **13**, 4087–4095
13 Kerppola, T. K., Luk, D. and Curran, T. (1993) Mol. Cell. Biol. **13**, 3782–3791
14 Schule, R., Rangarajan, P., Kliewer, S., Ransone, L. J., Bolado, J., Yang, N., Verma, I. M. and Evans, R. M. (1990) Cell **62**, 1217–1226
15 Schule, R. and Evans, R. M. (1991) Trends Genet. **7**, 377–381
16 Schienman, R. I., Gualberto, A., Jewell, C. M., Cidlowski, J. A. and Baldwin, Jr., A. S. (1995) Mol. Cell. Biol. **15**, 943–953
17 Mukaida, N., Mahe, Y. and Matsushima, K. (1990) J. Biol. Chem. **265**, 21128–21133
18 Stein, B., Cogswell, P. C. and Baldwin, Jr., A. S. (1993) Mol. Cell. Biol. **13**, 3964–3974
19 Scheinman, B. I., Cogswell, P. C., Lofquist, A. K. and Baldwin, A. S. (1995) Science **270**, 283–286
20 Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A. and Karin, K. (1995) Science **270**, 286–290
21 Butcher, E. C. (1991) Cell **67**, 1033–1036
22 Whelan, J. (1996) Trends Biochem. Sci, **21**, 65–70
23 Lewis, H., Kaszubska, W., Delamarter, J. and Whelan, J. (1994) Mol. Cell. Biol. **9**, 5701–5709
24 Kaszubska, W., Hooft van Huijsduijnen, R., Ghersa, P., DeRaemy-Schenk, A.-M., Chen, P. C., Hai, T., Delamarter, J. and Whelan, J. (1993) Mol. Cell. Biol. **13**, 7180–7190
25 Cronstein, B. N., Kimmele, S. C., Levin, R. I., Martiniuk, F. and Weissman, G. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 9991–9995
26 Collins, T. A., Williams, G. I., Johnston, G. I., Kim, J., Eddy, R., Shows, T., Gimbrone, M. A. and Bevilaqua, M. P. (1991) J. Biol. Chem. **266**, 2466–2473
27 Whelan, J., Ghersa, P., Hooft van Huijsduijnen, R., Gray, J., Chandra, G., Talabot, F. and Delamarter, J. F. (1991) Nucleic Acids Res. **19**, 2645–2653
28 Wingfield, P., Payton, M., Tavernier, J., Barnes, M., Shaw, A., Rose, K., Simona, M. G., Demczuk, S., Williamson, K. and Dayer, J.-M. (1986) Eur. J. Biochem. **160**, 491–498
29 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30 Van den Hoff, M. J. B., Moorman, A. F. M. and Lamers, W. H. (1992) Nucleic Acids Res. **20**, 2902
31 Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) Nucleic Acids Res. **11**, 1475–1489
32 Guesdon, F., Ikebe, T., Stylianou, E., Warwick-Davies, J., Haskill, S. and Saklatvala, J. (1995) Biochem. J. **307**, 287–295
33 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
34 Ray, K. P. and Searle, N. (1994) Agents Actions **38**, C61–C63
35 Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.-C., Heyman, R. A., Rose, D. W., Glass, C. K. and Grosenfeld, M. G. (1996) Cell **85**, 403–414
36 Perkins, N. D., Felzien, L. K., Bette, J. C., Leung, K., Beach, D. H. and Nabel, G. J. (1997) Science **275**, 523–527
37 Brostjan, C., Anrather, A., Csizmadia, V., Natarajan, G. and Winkler, H. (1997) J. Immunol. **158**, 3836–3844

Received 29 May 1997/13 August 1997; accepted 26 August 1997