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Effects of dexamethasone on cytokine and phorbol ester stimulated c-Fos and c-Jun DNA binding and gene expression in human lung

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Effects of dexamethasone on cytokine and phorbol ester stimulated c-Fos and c-Jun DNA binding and gene expression in human lung. I.M. Adcock, C.R. Brown, H. Shirasaki, P.J. Barnes. ©ERS Journals Ltd 1994.
ABSTRACT: Glucocorticosteroids have a wide variety of effects which result in the long-term dampening of inflammatory responses. An important site of steroid action may be on the control of the activator protein-1 (AP-1) binding to deoxyribonucleic acid (DNA). AP-1 is a proinflammatory transcription factor composed of a heterodimer of Fos and Jun proto-oncogenes, which can be induced by phorbol esters and various cytokines. We have examined the hypothesis that dexamethasone may inhibit inflammation *via* an effect on AP-1 activation in human lung tissue.

The effect of dexamethasone on the phorbol ester and cytokine activation of AP-1 and its monomers was examined in human lung tissue obtained from transplantation donors. AP-1 activation was measured by its ability to bind DNA, its localization in the nucleus by Western blotting, and the levels of *fos* and *jun* messenger ribonucleic acids (mRNAs) using Northern blotting.

The phorbol ester, phorbol myristate acetate (PMA), caused a significant 2-3 fold increase in AP-1 DNA binding, which was sustained for 24 h and completely attenuated by co-incubation with dexamethasone. Dexamethasone alone caused a 40% decrease in AP-1 DNA binding. Dexamethasone modulated the expression of both *c-jun* and *c-fos* mRNA and produced long-term (24 h) 40% reduction in both mRNAs when compared to control tissues. PMA induced a rapid and prolonged increase in c-Fos and c-Jun nuclear localization, which was not attenuated by co-incubation with dexamethasone. Levels of c-Fos and c-Jun protein, within the nucleus and cytoplasm were unchanged for up to 3 h following dexamethasone treatment alone, but were greatly reduced at 24 h.

This suggests that in human lung, the short-term effect of glucocorticoids is to prevent AP-1 DNA binding within the nucleus *via* protein-protein interactions. In the long-term, glucocorticoids reduce the level of Fos and Jun available for AP-1 formation. This may be an important molecular mechanism of steroid action in asthma and other chronic inflammatory lung diseases.
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Inhaled glucocorticosteroids are the most effective therapy currently available for the treatment of asthma. Despite their efficacy in controlling all types of asthma, their mode of action remains uncertain. Glucocorticoids inhibit many steps in the inflammatory process, and limit the proliferative responses of cells in chronic destructive diseases [1]. In general, steroids appear to dampen the defence reactions activated by challenges to homeostasis. In asthma, inhaled steroids reduce airway hyperresponsiveness and suppress the inflammatory response in asthmatic airways [2]. Recent reports have indicated that glucocorticoid receptors (GR) antagonize the actions of many inflammatory mediators, which act through various second messenger systems that all converge on the transcription factor, activator protein-1 (AP-1), which is a heterodimer of the proto-oncogene proteins c-Fos and c-Jun [3]. This suggests that an important site of steroid action may be on the control of transcription factor binding to specific *cis*-acting elements on deoxyribonucleic acid (DNA). These *trans*acting proteins affect the rate of messenger ribonucleic acid (mRNA) synthesis by binding to discrete sequences of promoters in the 5' upstream controlling region of genes [4]. Interaction of distinct regulatory pathways may occur *via* cross-coupling of transcription factors [5–7]. Cytokines, such as tumour necrosis factor-α (TNFα), have been implicated in the pathogenesis of many chronic inflammatory diseases, including arthritis and asthma [8], and their molecular effects include the induction of several specific transcription factors, including AP-1. Protein kinase C (KPC)

may also be involved in chronic regulation of cell function [9] and may be activated by phorbol esters, such as phorbol myristate acetate (PMA). PKC activation by phorbol esters also activates AP-1 [10].

We have, therefore, examined in human lung the interaction of glucocorticoids with activated AP-1 and its components the proto-oncogenes, c-Fos and c-Jun.

Materials and methods

All chemicals used were of at least Analar grade and all solutions and glassware were sterilized either by autoclaving or by dry heat. Enzymes were supplied by Northumberland Biologicals Ltd (Washington, UK) and Promega (Southampton, UK). [^{32}P] γ-adenosine triphosphate (γATP) (3,000 Ci·mmol^{-1}) was purchased from Amersham International (Amersham, UK).

Lung tissue was obtained from 13 donors (aged 7–45 yrs) undergoing cardiac transplantation, who were maintained on ventilation prior to transplantation.

Molecular probes

C-*fos* and c-*jun* oligonucleotide probes were obtained from British Biotechnology (Oxford, UK). Probes were 5'-end labelled using [^{32}P] ATP and T_4 polynucleotide kinase to >10^9 counts per minute (cpm)·µg^{-1}. A 33 base pair (bp) oligonucleotide (5'-ACAGACCTGTTATTGCTCAATCTCGGCTGGCTG-) specific for 18S RNA was used as control.

Electrophoretic mobility shift assays (EMSA)

Nuclear proteins were extracted from chopped human lung tissue (5–10 mm^3) that had been incubated in oxygenated Kreb's solution at 20°C containing 0.1 µM PMA, 10 pM TNFα or 10 pM interleukin-2 (IL-2), in the presence or absence of 1 µM dexamethasone, according to the method of Osborn *et al*. [11]. The effect of various doses of dexamethasone (0.1 nM–10 µM) on the TNFα (10 pM) and the PMA (0.1 µM) stimulated AP-1 activation was also investigated. Tissue was lysed using nonidet P-40 (NP-40) at 4°C for 15 min and soluble nuclear extracts obtained following osmotic lysis of the nuclear envelope. Nuclear proteins (10 µg) were incubated with 50,000 cpm ^{32}P-labelled double-stranded (ds) oligonucleotides containing a tandem repeat of the consensus sequence for the AP-1 DNA binding site (TRE) (-GATCCTTCG**TGACTCA**GCGGGATCCTTCG**TGACTCA**GCGG-) (Gibco-BRL, Uxbridge, UK) as described by Schüle *et al*. [5]. DNA-protein complexes were resolved on a 6% non-denaturing polyacrylamide gel (37:1 acrylamide:bis-acrylamide) in 0.25× Tris-Borate-ethylenediamine tetra-acetic acid (EDTA) buffer (TBE). Gels were dried and autoradiographed at -70°C using Kodak XAR-1 film. The retarded bands were quantified by laser densitometry (Protein Databases Inc., New York, USA) and band density measurements were then expressed as a percentage of binding at t=0. Specificity was determined by addition of a 100 fold excess unlabelled ds AP-1 oligonucleotide. In order to confirm the identity of the components in the retarded complexes, supershift experiments were conducted. In these experiments, antibodies (1–5 µg) to human c-Fos, c-Jun (Serotec, Oxford, UK) or GR (Cambridge Bioscience, Cambridge, UK) were added to the reaction mix after addition of labelled oligonucleotide, and incubated at 20°C for 60 min before the samples were loaded onto gels.

RNA extraction and Northern blotting

Lung tissue was chopped into 0.5 cm^3 cubes and incubated in oxygenated Kreb's solution at room temperature for up to 24 h in the presence or absence of 1 µM dexamethasone. Total RNA was extracted from lung parenchyma according to the method of Chomczynski and Sacchi [12], and poly(A+) mRNA was isolated as described previously [13]. RNA was size fractionated by agarose gel electrophoresis, transferred to nylon membranes and cross-linked by UV irradiation (Stratalinker, Stratagene, Cambridge, UK). RNA transcripts were detected using 10^7 cpm of either a c-*fos* or a c-*jun* oligonucleotide probe at 42°C in hybridization buffer [14]. Filters were autoradiographed for 7–10 days and analysed by laser densitometry. Filters were stripped and reprobed with an oligonucleotide directed against 18S ribosomal RNA for comparison of RNA loading.

Western blotting

Nuclear proteins were extracted from chopped human lung tissue (5–10 mm^3) that had been incubated in oxygenated Kreb's solution at 20°C containing 0.1 µM PMA, in the presence or absence of 1 µM dexamethasone, according to the method of Osborn *et al*. [11]. Two hundred micrograms of nuclear protein were size fractionated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transblotted onto nitrocellulose-enhanced chemiluminescence (ECL) membranes (Amersham International, Amersham, UK). Membranes were blocked overnight with 2% casein, before incubation with 1:1000 sheep anti-human c-Fos or anti-human c-Jun antibody (Serotec) at 18°C for 3 h. After extensive washing, bound antibody was detected using 1:2000 rabbit anti-sheep antibody ($F(ab')_2$ fragment) linked to horseradish peroxidase. After further washing, the bound antibody complexes were detected using enhanced chemiluminescence (Amersham International, Amersham, UK) [15].

Data analysis

All data are expressed as mean±standard error (SEM). Results between treatment groups were compared by analysis of variance (ANOVA). At specific time-points, results were compared by Wilcoxons nonparametric analysis, and a value of $p<0.05$ was considered significant.

Results

Transcription factor activity

Band shift assays on nuclear extracts showed a marked increase in AP-1 DNA binding activity following PMA treatment, a reduction in band intensity with dexamethasone treatment and a reduction in the PMA-induced increase following simultaneous dexamethasone treatment. There was no change in the levels of AP-1 binding in control untreated lung tissue (fig. 1a). Quantification of these and other similar results by laser densitometry showed a 210±68% increase in AP-1 activity present in the lung within 15 min of incubation with PMA (0.1 µM) (fig. 1b). This enhancement was reduced to 92±18% at 30 min, and was sustained at around this level for the remainder of the experiment. Dexamethasone (1 µM) caused a marked decrease (40–55%) in specific AP-1 activity at all time-points studied. Combined treatment with PMA and dexamethasone significantly reduced (70–80%; $p<0.01$) this enhancement in AP-1 binding within 15 min, with levels returning to those seen in control lung within 30 min (fig. 1b). The ability of TNFα and IL-2 to induce AP-1 DNA binding was also determined. TNFα (10 pM) and IL-2 (10 pM) produced, respectively, a 121±19% and an 85±8% increase in

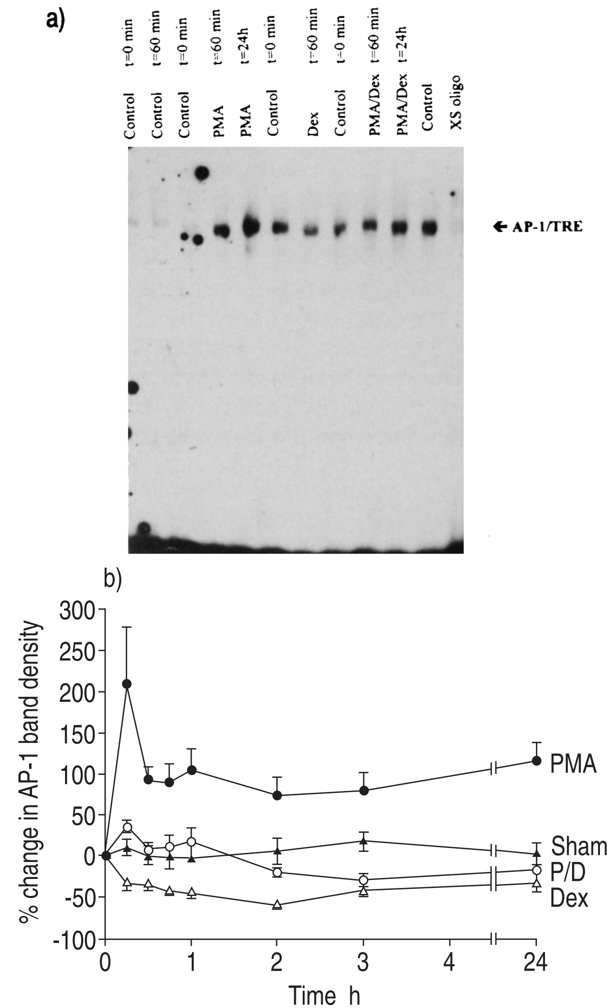

Fig. 1. – a) Gel mobility assay showing an increase in activator protein-1 (AP-1) binding after treatment with phorbol myristate acetate (PMA) (0.1 µM) over time, a decrease following dexamethasone (Dex) treatment (1 µM), and a reduced induction of AP-1 binding after combined treatment of human lung parenchyma. The arrow indicates the position of AP-1 bound to the ^{32}P-labelled specific AP-1 consensus deoxyribonucleic acid (DNA) binding sequence (TRE) (TRE/AP-1) which has been retarded in the gel matrix. Specificity of binding is shown by incubation of nuclear cell extract with and without excess unlabelled oligonucleotide (XS oligo) on the amount of AP-1 present. In all experiments 10 µg human lung nuclear extract was used. b) Time course of transcription factor binding. Effect of PMA (0.1 µM) treatment on AP-1 binding in the absence (●) or presence (❍) of dexamethasone, 1 µM, (P/D) in human lung tissue over 24 h. The effects of dexamethasone (Dex), 1 µM, (∆) and control media (Sham) treatment (▲) on AP-1 binding are also shown. Data are presented as the mean±SEM of 6–12 observations.

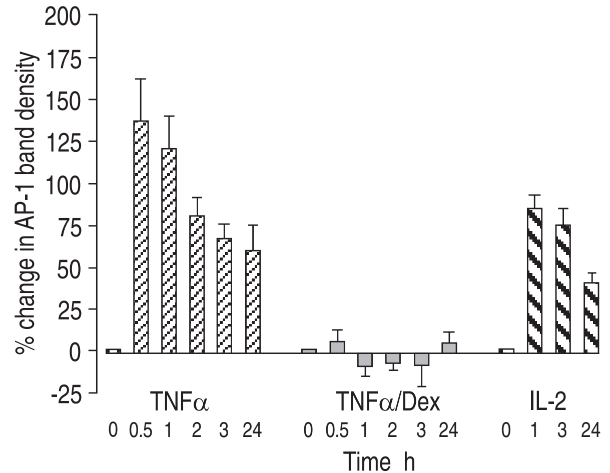

Fig. 2. – Time-course of the change in AP-1 DNA binding in human lung following treatment of tissue with 10 pM tumour necrosis factor-α (TNFα) or 10 pM interleukin-2 (IL-2). The effect of 1 µM dexamethasone (Dex) on the ability of 10 pM TNFα to induce AP-1 binding is also shown in the central panel. The amount of AP-1 binding in all cases was compared to the level seen at t=0 which was assigned a value of 0. Data are presented as mean±SEM of 3–8 observations. ▭: TNFα; ▭: TNFα/Dex; ▭ : IL-2. For abbreviations see legend to figure 1.

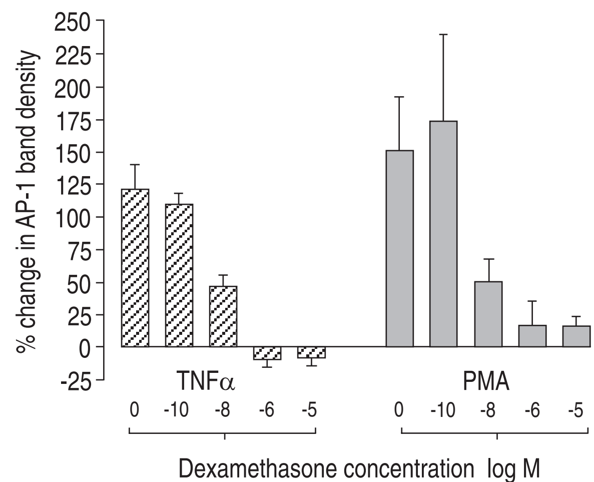

Fig. 3. – Dose response of dexamethasone on TNFα (10 pM) and PMA (0.1 µM) induced AP-1 DNA binding in human lung tissue after 1 h of incubation. The concentration of dexamethasone used is indicated beneath each bar. The data are presented as the mean±SEM of 4–8 observations. ▭: TNFα; ▭ : PMA. For abbreviations see legends to figures 1 and 2.

AP-1 binding after 1 h of treatment. This increased level of AP-1 binding was reduced over time, but remained at a higher level than in controls for the duration of the experiment (24 h) (fig. 2). There was a dose-dependent effect of dexamethasone on TNFα and PMA-induced AP-1 binding that was seen to be maximal at 10^{-6} M (fig. 3). Dexamethasone (1 µM) greatly attenuated the increase in AP-1 DNA binding induced both by TNFα and PMA over the whole treatment time (figs 1b and 2).

When antibodies to specific proteins involved in DNA/protein complexes are incubated with proteins in electrophoretic mobility shift assays, the retarded DNA/protein complex may either be further retarded in the gel (supershifted) or the intensity of the retarded band greatly reduced depending on the site of antibody interaction. The antibodies used in the supershift experiments; anti-c-Fos, anti-c-Jun and anti-GR, were directed against epitopes at or close to their respective DNA binding sites. As a result, there were no supershifted bands seen on the autoradiographs. Binding of the c-Fos and c-Jun antibodies to the AP-1 complex produced a reduction in the band intensity due to inhibition of AP-1/dsDNA binding (fig. 4). The anti-GR antibody produced similar results to that seen with the c-Fos and c-Jun antibodies (fig. 4).

c-jun/c-fos mRNA

Northern blot analysis of *c-jun* mRNA indicated the presence of a single band at 2.8 kb, which was modulated in the presence of 1 µM dexamethasone in a biphasic manner. *C-jun* mRNA levels rose to 136±59% of basal expression at 1 h, falling to -65±21% of control levels at 3 h and remaining reduced for up to 24 h (fig. 5a). *C-fos* mRNA gave a single band on Northern analysis at 2.4 kb, which was modulated by dexamethasone

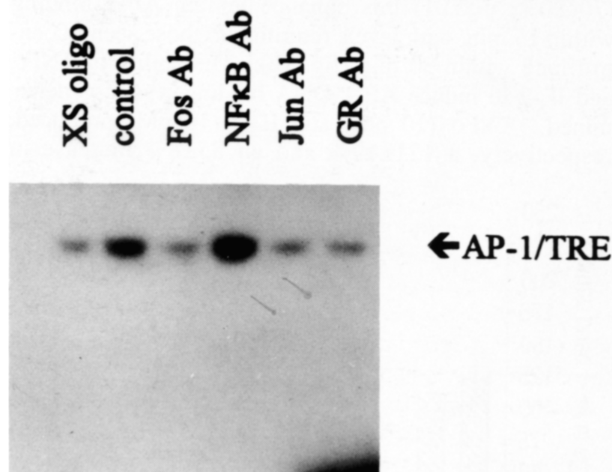

Fig. 4. – Supershift assay showing effect of an anti-c-Fos, c-Jun or anti-GR antibody on an AP-1 electrophoretic mobility shift assay. Lane 2 shows the level of AP-1 binding to the specific AP-1 consensus sequence, TRE (AP-1/TRE) in untreated lung. The effect of excess unlabelled AP-1 double-stranded (ds) oligo is shown in lane 1. The AP-1/TRE complexes are greatly diminished when compared to control lanes when the proteins were incubated with anti-c-Fos antibody (lane 3), anti-c-Jun antibody (lane 5) or anti-GR antibody (lane 6). The control anti-nuclear factor kappa B (NFκB) antibody has no effect on AP-1/TRE binding (lane 4). The specific AP-1/TRE band is indicated by an arrow. GR: glucocorticoid receptor; Ab: antibody; AP-1: activator protein-1; TRE: TPA responsive element; TPA: 12-O-tetra decanoylphorbol 13-acetate; XS oligo: excess unlabelled oligonucleotide.

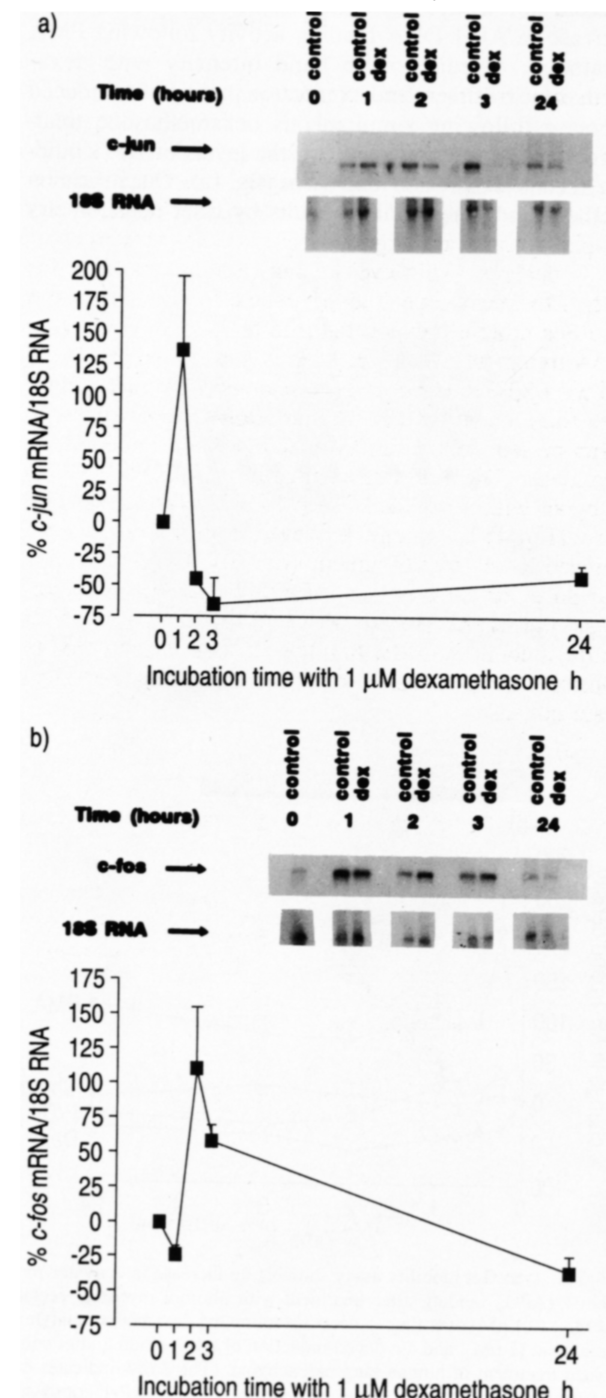

Fig. 5. – a) Northern blot analysis of *c-jun* messenger ribonucleic acid (mRNA) following dexamethasone treatment in human lung. Upper panel shows *c-jun* bands (arrowed) and middle panel 18S RNA bands as a control for gel loading. Lower panel shows the percentage change in the ratio of densitometric analysis of the bands above. Values plotted are means±SEM (n=8). b) Northern blot analysis of *c-fos* mRNA following dexamthasone treatment in human lung. Upper panel shows *c-fos* bands (arrowed) and middle panel 18S RNA bands as a control for gel loading. Lower panel shows the percentage change in the ratio of densitometric analysis of the bands above. Values plotted are means±SEM (n=8).

in a time-dependent manner. *C-fos* mRNA expression was reduced to -25±6% of baseline levels at 1 h; however, levels increased to greater than those of control at 2 h (+110±45%) and 3 h (+59±11%), and were then reduced to -34±12% of control levels by 24 h (fig. 5b).

c-Fos and c-Jun protein expression

Western blot analysis of c-Fos and c-Jun protein in nuclear extracts from these samples detected both Fos and Jun proteins. Following PMA treatment, there was a rapid (15 min) and large increase in c-Fos protein within the nucleus. This level remained above baseline for 24 h. Dexamethasone (1 µM) had no effect on the amount of c-Fos protein translocated into the nucleus following PMA treatment, except at 24 h, when the levels detected were 80% lower than those seen in PMA treated samples (data not shown). Similar results were seen with c-Jun protein following PMA and PMA/dexamethasone treatment. There was no change in expression in c-Fos or c-Jun within the nucleus up to 3 h after dexamethasone (1 µM) treatment; however, levels were greatly reduced 24 h after treatment (fig. 6). Dexamethasone had no effect on c-Fos or c-Jun levels within the cytoplasm during this period. PMA treatment had no effect on the amount of GR within the nucleus as compared to control, untreated tissue over the time course studied (data not shown).

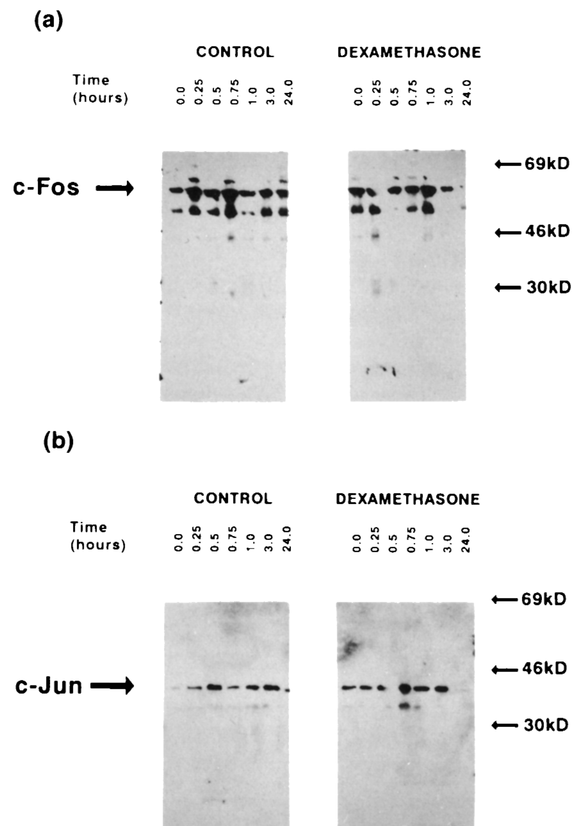

Fig. 6. – Western blot analysis of: a) c-Fos; and b) c-Jun protein from 200 µg nuclear extract isolated from dexamethasone treated and untreated human lung over a 24 h time course. Specific bands are indicated by arrows. Molecular weight markers are as indicated.

Discussion

The control of chronic inflammation is important in several inflammatory conditions, including asthma and fibrosing lung diseases. The regulatory pathways that control chronic inflammation are complex and multifactorial. Glucocorticoids inhibit the inflammatory response of many cells to a wide variety of stimuli, and so an understanding of their mode of action is of central importance. Cytokines may be important mediators of chronic inflammation. Although the molecular pathways involved in cytokine action are not well understood, several cytokines act on responsive cells to induce the activation or synthesis of a few well-characterized transcription factors. The finding that TNFα and IL-2 induced a rapid long-lasting activation of AP-1 in the human lung, which is mimicked by PMA, and is inhibited by dexamethasone in a dose-dependent manner, suggests that this may be an important molecular mechanism of steroids. This could account for the multiple anti-inflammatory actions of glucocorticosteroids and for their efficacy in chronic inflammatory diseases.

Several mechanisms have been proposed to account for interaction between transcription factors. One factor could replace another at its site of action on the upstream regulatory site of the target genes, the two factors may compete for another factor at either the gene or protein level, or there may be a direct interaction between the GR and other transcription factors at a protein-protein level [16]. Direct protein-protein interaction is more likely to account for the results of our study, at least in the short-term, as AP-1 binding is reduced in the nuclear extract, although no reduction in the components of AP-1 is seen in the same extracts by Western blotting until 24 h. Furthermore, the results of the antibody binding experiments suggest a direct interaction between AP-1 and the glucocorticoid receptor within this tissue.

Glucocorticoids have been found to inhibit the synthesis of several cytokines, including IL-1 and TNFα [17, 18]. Although steroids may inhibit the inflammatory process at several sites, the direct interaction with transcription factors, such as AP-1, may be of particular relevance, since this could provide a mechanism for switching off inflammation driven by cytokines known to activate this transcription factor [19].

DIAMOND *et al.* [20] have proposed an alternative model for AP-1/GR interaction in the nucleus, involving overlapping atypical glucocorticoid response elements (GREs) and AP-1 binding sites (12-O-tetradecanoylphorbol-13-acetate (TPA) responsive element, TRE) termed a composite GRE. C-Jun homodimers interact with GR to cause a synergistic effect, increasing transcription of responsive genes, whilst c-Fos/c-Jun heterodimers interact with DNA bound GR to switch off transcription. Thus, transcription rate is controlled by the level of c-Fos in the cell.

C-Jun upregulates its own synthesis *via* binding to its own TRE, whilst c-Fos is known to be negatively controlled by AP-1 binding to a TRE in its 5' promoter region [3]. Our studies have demonstrated that glucocorticoids

have a biphasic effect both on *c-fos* and *c-jun* mRNAs. *c-jun* mRNA levels were induced at 1 h before falling to much reduced levels at 3 h. In contrast, *c-fos* mRNA levels were reduced at 1 h, increased to 110% more than control levels by 2 h, and were reduced to lower levels again by 24 h. This is similar to results described in previous studies, which have shown an induction of *c-fos* mRNA with glucocorticoid treatment [7, 21, 22]. Suppression of *c-fos* and *c-jun* expression is due to a rapid breakdown of mRNAs, both of which are known to contain adenine-uracil (AU)-rich regions in their 3' untranslated regions, and are thus susceptible to rapid ribonuclease (RNase) action. Glucocorticoids have been shown to affect mRNA degradation by binding to 3' regions of mRNAs without having a direct effect on transcription [23], and may alter *c-jun* and *c-fos* mRNA levels by directly binding to the 3' tail of their respective mRNAs. Alternatively, *c-fos* and *c-jun* transcription may be affected by glucocorticoid receptor binding to AP-1. The repression of *c-jun* mRNA expression due to glucocorticoids in the human lung may be mediated through the interaction between the GR and the AP-1 complex, through protein-protein interactions preventing DNA binding of either transcription factor to the *c-jun* promoter region, since no GRE has been found within the *c-jun* upstream regulatory sequence [3]. By the same mechanism, the opposite effect would be expected on *c-fos* mRNA.

The difference in time course between the two components of AP-1 may have functional significance, in that *c-fos* levels regulate the transcriptional activity of the AP-1 Fos/Jun heterodimer. This difference in results may be resolved by expanding the time course to beyond 3 h and by increasing the number of time-points between 0 and 1 h, although other studies suggest that GR effects on *c-jun* expression are highly variable, depending on the cell types used [6, 23]. It is also possible that GR controls the expression of a *c-fos* regulatory protein or its phosphorylation, thereby affecting *c-fos* transcription of mRNA half-life.

The results of our studies indicate that in human peripheral lung tissue, nuclear localization and DNA binding of the transcription factor AP-1 is induced by phorbol esters in a time-dependent manner. Gel mobility shift assays indicate that the activated GR functionally interacts with these transcription factors within the nucleus by inhibition of DNA binding, and not by prevention of translocation into the nucleus, and may thereby regulate the induction of various long-term inflammatory mediators, such as cytokines, or by agents that may activate protein kinase C.

The anti-inflammatory properties of glucocorticoids in lung could be explained if glucocorticoids repressed cytokine-induced AP-1 mediated induction of mRNA coding for other cytokines, enzymes and receptor genes. Cross-coupling of distinct and opposing regulatory pathways provides another level of regulation in determining the inflammatory response of the cell. This inhibition of transactivation may be an important molecular site of steroid action in asthma and other chronic inflammatory diseases.

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