p65-activated histone acetyltransferase activity is repressed by glucocorticoids: Mifepristone fails to recruit HDAC2 to the p65/HAT complex

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Running Title: Full transrepression by dexamethasone requires HDAC recruitment
Summary

Glucocorticoids acting through their specific receptor can either enhance or repress gene transcription. Dexamethasone represses IL-1β-stimulated histone acetylation and GM-CSF expression through a combination of direct inhibition of p65-associated histone acetyltransferase (HAT) activity and by recruiting histone deacetylase 2 (HDAC2) to the p65/HAT complex. Here we show that mifepristone, a glucocorticoid receptor partial agonist, has no ability to induce gene expression but represses IL-1β-stimulated histone acetylation and GM-CSF release by 50% maximally. Mifepristone was able to inhibit p65-associated HAT activity to the same extent as dexamethasone but failed to inhibit the natural promoter to an equal extent due to an inability to recruit HDAC2 to the p65-associated HAT complex. This data suggests that the maximal repressive actions of glucocorticoids require recruitment of HDAC2 to a p65/HAT complex. This data also suggests that pharmacological manipulation of specific histone acetylation status is a potentially useful approach for the treatment of inflammatory diseases.

Key Words: glucocorticoid receptor / histone acetylation / HDAC2 / Mifepristone / transrepression
Introduction

Actively transcribed genes are associated with an unwinding of the previously closed DNA structure allowing accessibility to DNA-binding proteins, thereby allowing modulation of gene transcription (1;2). DNA is packaged into chromatin, a highly organised and dynamic protein-DNA complex. The fundamental subunit of chromatin, the nucleosome, is composed of an octamer of 4 core histones; an H3/H4 tetramer and two H2A/H2B dimers, surrounded by 146 bp of DNA (1;3). The nucleosome therefore acts as a barrier to the initiation of transcription by preventing the access of transcription factors, and RNA polymerase II, to their cognate recognition sequences on DNA (4). The N-terminal tails of histones contain lysine residues that are the sites for post-transcriptional acetylation. This is a dynamic process that occurs on actively transcribed chromatin only (5). In addition, core histones may be modified by phosphorylation, methylation, ADP ribosylation or ubiquitinisation of specific amino acid residue (6).

Increased gene transcription is associated with an increase in histone acetylation, whereas hypo-acetylation is correlated with reduced transcription or gene silencing (2;7). Targeted acetylation of histone H4 tails plays an important role in allowing regulatory proteins to access DNA and is likely to be a major factor in the regulation of gene transcription (8-10).

Glucocorticoids are the most effective therapy for the treatment of inflammatory diseases such as asthma, a chronic inflammatory disease of the airway (11). Functionally, they act partly by inducing some anti-inflammatory genes such as secretory leukocyte proteinase inhibitor (SLPI)(12), Lipocortin-1 (13) and IL-1 receptor antagonist (14) but mainly by repression of inflammatory genes, such as cytokines, adhesion molecules, inflammatory enzymes and receptors (11). They act by binding to a cytosolic glucocorticoid receptor (GR), which upon
binding is activated and rapidly translocates to the nucleus. Within the nucleus, GR either induces gene transcription by binding to specific DNA elements in the promoter/enhancer regions of responsive genes or reduces gene transcription by transrepression (15). GR reduces gene transcription by a functional interaction with pro-inflammatory transcription factors such as AP-1 (Fos:Jun heterodimers) and NF-κB (p65:p50 heterodimers) (15-17). We have recently shown that GR represses NF-κB-mediated HAT activity and GM-CSF release by a combination of direct inhibition of CBP-associated HAT activity, but not that of CBP itself, and by recruitment of HDACs to the NF-κB activation complex (18).

Many of the anti-inflammatory effects of corticosteroids may be mediated by repression of transcription factors (transrepression), whereas the endocrine and metabolic effects of corticosteroids are mediated via GRE binding (transactivation). This has led to a search for novel corticosteroids that selectively transrepress, thus reducing the risk of systemic side effects. A separation of transactivation and transrepression has been demonstrated using reporter gene constructs in transfected cells using selective mutations of GR (19). Furthermore, some corticosteroids, such as RU24858, RU486 (mifepristone) and ZK98299, have a greater transrepression than transactivation effect (19;20). These corticosteroids, including RU24858 and RU40066, have anti-inflammatory effects in vivo (21). These studies rely on the over-expression of components of these pathways, which could lead to problems in interpretation. We have therefore examined the role of CBP and associated HATs in the regulation of glucocorticoid functions in non-transfected cells.

We have investigated the ability of dexamethasone and mifepristone to suppress expression of the inflammatory cytokine, granulocyte-macrophage colony stimulation factor (GM-CSF) and induce SLPI, and to regulate histone acetylation and deacetylation in A549
cells. We have demonstrated that mifepristone, unlike dexamethasone, is unable to induce histone H4 acetylation but is able partially to repress IL-1β-stimulated histone acetylation without recruitment HDAC2 to the p65 complex. This results in a partial reduction of acetylated K8 associated with the GM-CSF promoter and reduced GM-CSF mRNA production. The mechanism of mifepristone repression of IL-1β-stimulated histone H4 K8 acetylation was by direct inhibition of p65-associated HAT activity with no effect on recruitment of HDAC2 to the p65/HAT complex.
Experimental Procedures

Cell Culture

A549 cells, a human lung adenoma cell line (ATCC designation CCL185) and a good model for human lung epithelial cells, were grown to 50% confluence in Dulbecco’s modified medium (DMEM) containing 10% foetal calf serum (FCS) before incubation for 48-72 hr in serum-free media. Cells were stimulated by IL-1β (1ng/ml) and the effects of dexamethasone, mifepristone and of the histone deacetylase inhibitor trichostatin A (TSA) (Sigma, Poole, UK) on GM-CSF and SLPI release measured.

GM-CSF and SLPI ELISAs

Determination of GM-CSF expression was measured by sandwich ELISA (R&D Systems Europe, Abingdon, UK) according to the manufacturer’s instructions. For immunoassay of SLPI, polystyrene microtitre plates were coated overnight at 4°C with sample diluted with hydroxy carbonate (pH 9.6). Plates were blocked for 2 hr at room temperature with 5% ovalbumin in PBS. Antibodies against SLPI (R&D Systems Europe, Abingdon, UK) was diluted 1:2000 and added to each plate. After 1 hr at room temperature plates were washed sequentially with 0.1% Tween20-PBS and incubated with HRP conjugated goat anti-rabbit antibody (DAKO, Cambridge, UK) for 1 hr. Detection was performed following R&D instructions. Recombinant human SLPI (R&D Systems Europe) was used as a standard.

RNA isolation and RT-PCR.

PCR primers were as follows with annealing temperature and product size in parentheses: GM-CSF sense 5’-CCC AAT gAA gCC TCA CCg AAT-3’ and antisense 5’-TCg gAg Cgg
gTA gTT AAC AgC-3’ (67°C, 604 bp); SLPI sense 5’-ATg AAg TCC AgC ggC CTC TT-3’ and antisense 5’-ATg gCA ggA ATC AAg CTT TC-3’ (54°C, 408 bp); GAPDH sense 5’-CCC TgA ATT TgA Cag TCT CACC-3’ and antisense 5’-CAC AAT AAA ACT TgC CCA gAA AAA-3’ (62°C, 175 bp). Extraction of RNA from A549 cells was performed using an RNeasy Mini Kit according to the manufacturer’s instructions (Qiagen, Crawley, UK). Sample RNA was quantified by spectrophotometry, and 1 µg was reverse transcribed to cDNA as previously described (23). PCR reactions were performed on 5 µl of the cDNA with a Hybaid Omnigene thermal cycler (Hybaid, Ashford, Middlesex, UK) in a final reaction volume of 25 µl in the presence of 0.4 U of Taq DNA polymerase. 35 cycles were used, with a denaturing step at 94°C for 45 s, followed by the specific primer annealing temperature for 45 s and an extension step at 72°C for 45s.

Direct histone extraction

Histones were extracted from nuclei overnight using HCl and H2SO4 at 4°C as previously described (18). Cells were microfuged for 5 min and the cell pellets extracted with ice-cold lysis buffer (10mM Tris-HCl, 50mM sodium bisulphite, 1% Triton X-100, 10mM MgCl2, 8.6% sucrose, complete protease inhibitor cocktail (Boehringer-Mannheim, Lewes, UK) for 20 min at 4°C. The pellet was repeatedly washed in buffer until the supernatant was clear (centrifuge at 8000rpm, 5min after each wash) and the nuclear pellet washed in nuclear wash buffer (10mM Tris-HCl, 13mM EDTA) and resuspended in 50µl of 0.2N HCl and 0.4N H2SO4 in distilled water. The nuclei were extracted overnight at 4°C and the residue microfuged for 10 min. The supernatant was mixed with 1ml ice-cold acetone and left overnight at -20°C. The
sample was microfuged for 10 min, washed with acetone, dried and diluted in distilled water. Protein concentrations of the histone containing supernatant were determined by Bradford protein assay kit (BioRad, Hemel Hempstead, UK).

**Western blotting**

Immunoprecipitates, whole cell extractions or isolated histones were measured by SDS-PAGE and Western blot analysis using ECL (Amersham, Amersham, UK). Proteins were size-fractionated by SDS-PAGE and transferred to Hybond-ECL membranes. Immunoreactive bands were detected by ECL.

**Immunocytochemistry**

A549 cells (0.5 x 10^6) were cultured in 8 well slide chambers with mifepristone (10^-6 M), dexamethasone (10^-6 M) or IL-1β (1 ng/ml). Cells were washed with Hanks solution, and air-dried for 30 min at RT. Cells were then fixed in ice-cold acetone-methanol (50/50, w/w) (-20°C) for 10 min. Slides were air dried and incubated with blocking buffer (20% normal swine serum in PBS, 0.1% saponin)(Dako) for 20 min, followed by 1 hr incubation with primary antibody solution (PBS, 0.1% saponin, 1% BSA). Antibodies against acetylated H4-K5, H4-K8 (Serotec) (24) glucocorticoid receptor (GR) (Serotec) and NF-kB:p65 (Santa Cruz Biotech. Inc., Santa Cruz, CA) were used at 1:100 to 1:300 dilution. Slides were washed twice and incubated with biotinylated swine anti-rabbit IgG (Dako)(1:200) for 45 min. Slides were washed again before incubation with fluorescein isothiocyanate-conjugated streptavidine (1:100) for 45 min. The slides were washed twice more before counterstaining with 20% haematoxylin, and mounting. Stained cells were observed by fluorescent microscopy.
Histone acetylation activity

Cells were plated at a density of 0.25 x 10^6 cells/ml and exposed to 5µCi/ml of [3H] acetate (NEN life science). After incubation for 10 min at 37°C cells were stimulated for 6 hr. Histones were isolated and separated by electrophoresis on SDS-16% polyacrylamide gel. Gels were stained with Coommasie brilliant blue and the core histones (H2A, H2B, H3 and H4) excised. The radioactivity in extracted core histones was determined by liquid scintillation counting and normalised to protein level.

Histone deacetylation assay

Radiolabelled histones were prepared from A549 cells following incubation with TSA (100ng/ml, 6hr) in the presence of 0.1 mCi/ml [3H]-acetate. Histones were dried and resuspended in distilled water. Crude HDAC preparations were extracted from total cellular homogenates with Tris-based buffer (10 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol) as previously reported (25). The crude HDAC preparation or immunoprecipitates were incubated with [3H]-labelled histone for 30 min at 30°C before the reaction was stopped by the addition of 1N HCl/0.4N acetic acid. Released [3H]-labelled acetic acid was extracted by ethylacetate and the radioactivity of the supernatant was determined by liquid scintillation counting.

Immunoprecipitation

Extracts were prepared using 100 µl of mild IP buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, complete protease inhibitor cocktail (Boehringer-Mannheim). The lysis
mixture was incubated on ice for 15 min and microfuged for 10 min at 4°C. Extracts were precleared with 20 µl of A/G agarose (a 50:50 mix; Santa Cruz Biotech Inc.) and 2 µg of normal rabbit IgG. After microcentrifugation, 20 µl of A/G agarose conjugated with 5µg of p65 antibody were incubated for 6 hours at 4°C with rotation. The immune complexes were pelleted by gentle centrifugation and washed 3 times with 1ml of IP buffer. For the HAT assay, immunoprecipitates were washed twice with IP-HAT buffer, and for Western blotting, after final wash with IP buffer, the buffer was aspirated completely and resuspended in Laemmli buffer.

**IP-HAT assays**

IP-HAT assays were performed using a modified method of Ogryzko (26). Immune complexes with resin were resuspended in 150 µl of HAT buffer (50mM Tris-HCl, pH 8.0, 10% glycerol, 1mM dithiothreitol, 0.1mM EDTA, complete protease inhibitor cocktail). Typically, 20 µl of free core histone solution extracted from A549 cells (final concentration 10 µg) and 30 µl of immunoprecipitate were incubated. Reactions were initiated by the addition of 0.25 µCi of [3H] acetyl-CoA (5Ci/mmol)(Amersham) and performed for 45 min at 30°C. After incubation, the reaction mixture was spotted onto Whatman p81 phosphocellulose filter paper (Whatman) and washed for 30 min with 0.2M sodium carbonate buffer (pH 9.2) at room temperature with 2-3 changes of the buffer, then washed briefly with acetone. The dried filters were counted in a liquid scintillation counter.

**Chromatin Immunoprecipitation (ChIP) Assay**

A-549 cells pre-treated for 30 min with dexamethasone or mifepristone were treated with IL-
1β (1 ng/ml) for 4 hr. Protein-DNA complexes were fixed by formaldehyde (1% final concentration) and treated as previously described (27). Cells were resuspended in 200 µl of SDS lysis buffer (50 mM Tris; pH 8.1, 1% SDS, 5 mM EDTA, complete proteinase inhibitor cocktail) and subjected to 3 steps with 10-sec pulses sonication on ice. Sonicated samples were centrifuged to spin down cell debris and the soluble chromatin solution were immunoprecipitated using sonicated salmon sperm DNA agarose A slurry (Upstate Biotechnology, Buckingham, UK) as described by Chen et al. (28). Protein-bound immunoprecipitated DNA was washed with LiCl wash buffer and TE, and immune-complexes were eluted by adding elution buffer (1% SDS, 0.1M NaHCO₃). The elution was treated successively for 4 hr at 65°C in 200 mM NaCl/1% SDS to reverse crosslinks and incubated for 1 hr at 45°C with 70 µg/ml Proteinase K (Sigma). DNA extracted with phenol/chloroform, precipitated with ethanol/0.3M NaHCOOH/20 µg glycogen, and resuspended in 50 µl of TE. Quantitative PCR was performed with 10 µl of DNA sample and 30 cycles. Primer pairs of GM-CSF were; forward 5-CTGACCACCTAGGGAAAAGGC-3, reverse 5-CAGCCACATCCTCCTCCAGAGAAC-3. PCR products were resolved by 3% agarose-gel and visualised with ethidium bromide.

**Statistics**

Results are expressed as means ± standard error of the mean (SEM). A multiple comparison was made between the mean of the control and the means from each individual treatment group by Dunnett’s test using SAS/STAT software (SAS Institute Inc., Cary, NC, USA). All statistical testing was performed using a two-sided 5% level of significance test. The concentrations of dexamethasone or mifepristone producing 50 % of the maximal inhibition
seen (EC$_{50}$) were calculated from concentration-response curves by linear regression.
Results

Effect of anti-glucocorticoid mifepristone on cytokine production and histone acetylation

We used the partial glucocorticoid agonist mifepristone (RU-486) to examine the different roles of GR transactivation and transrepression in control of GM-CSF and SLPI expression. Using reporter gene assays and over-expression of GR it has been postulated that mifepristone and related compounds may discriminate between transrepression and transactivation at GRE and AP-1 and NF-κB driven genes (19;20). Mifepristone inhibited IL-1β-stimulated GM-CSF release with a maximal inhibition of 50.4% (EC50 = 0.7 x 10^{-9} M, Figure 1a) and failed to induce SLPI expression (Figure 1b). This was in contrast to dexamethasone which totally suppressed IL-1β-stimulated GM-CSF release (EC50: 1.4 x 10^{-9} M) and caused a marked induction of SLPI release (7.6±0.5 ng/ml versus 1.5±0.7 at 10^{-6} M). Thus at the level of functional mediator release there is a clear discrimination between the activation and repressive roles of mifepristone.

In addition, mifepristone inhibited IL-1β-induced SLPI production with maximal inhibition of 65% at 10^{-5} M (Figure 1c). Dexamethasone strongly inhibited IL-1β-stimulated SLPI release (73%) at the concentration of 10^{-10} M before stimulating further SLPI release to levels seen with dexamethasone (10^{-6} M) alone. Many reports have suggested that control of gene expression is dependent upon changes in chromatin structure resulting from alterations in the acetylation status of core histones (29). We used the HDAC inhibitor trichostatin A (TSA, 10 ng/ml) to determine the role of HDACs in mediating the effects of dexamethasone and mifepristone on inhibition of IL-1β-stimulated GM-CSF release. TSA further stimulated
IL-1β-induced GM-CSF release suggesting a role for HDACs in modulating gene induction possibly reflecting a feedback mechanism to enable switching off of GM-CSF after an initial inflammatory pulse. In addition, TSA attenuated both the maximal inhibition and the EC50 for dexamethasone (maximum inhibition; 61% versus 91% at $10^{-6}$M, EC50; $7.8 \times 10^{-8}$M versus $1.4 \times 10^{-9}$M) whilst having no effect on mifepristone actions (maximum inhibition; 50% versus 45% at $10^{-6}$M, EC50; $0.7 \times 10^{-5}$M versus $1.2 \times 10^{-5}$M) (Figure 1d). This data suggests that mifepristone actions in repressing IL-1β-stimulated GM-CSF release are independent of HDAC activity whereas the full repressive effects of dexamethasone require HDAC activity.

**Mifepristone fails to induce histone acetylation but inhibits IL-1β-stimulated acetylation of bulk histone**

In order to determine whether dexamethasone or mifepristone could affect bulk IL-1β-stimulated histone acetylation, experiments were performed in whole cell extracts from cells treated with IL-1β and/or mifepristone or dexamethasone. IL-1β induced a 4-fold increase in histone acetylation (Figure 2). Mifepristone alone had no effect on basal histone acetylation but inhibited IL-1β-induced histone acetylation with maximal inhibition of 53% (Figure 2 upper panel). In contrast, dexamethasone had a biphasic effect on IL-1β-stimulated histone acetylation (Fig 2 lower panel) similar to the effects seen on SLPI production. Dexamethasone alone also induced histone acetylation in a concentration-dependent manner.

**Mifepristone induces GR nuclear translocation but fails to induce chromatin acetylation**
Immunofluorescence and confocal microscopy showed that dexamethasone and mifepristone enhanced GR nuclear translocation. IL-1β had no effect on GR translocation (Figure 3 a-d). In contrast to this result, IL-1β, but not dexamethasone and mifepristone, enhanced NF-κB (p65 subunit) nuclear translocation (Figure 3e-h). We examined histone H4 lysine (K) acetylation in order to confirm the role of histone acetylation in IL-1β, dexamethasone or mifepristone mediated effects. IL-1β caused acetylation of K8 (Figure 3p) and K12 residues (data not shown) whilst dexamethasone targeted acetylation on K5 (Figure 3k) and K16 (data not shown) as previously reported (18). Mifepristone failed to stimulate acetylation of any lysine residue (Figure 3 j, n).

**Mifepristone inhibits IL-1β-stimulated acetylation of histone H4 K8 but does not enhance H4-K5 acetylation**

Western analysis of specific acetylated lysines showed that dexamethasone, but not mifepristone, induced acetylation of K5 residues at the concentration of 10^{-6}M (Figure 4a). In addition, dexamethasone significantly inhibited IL-1β-stimulated K8 acetylation (Figure 4b). Mifepristone also reduced IL-1β-stimulated K8 acetylation but to a lesser extent than dexamethasone. This data suggests that mifepristone can slightly inhibit histone acetylation induced by IL-1β and, in contrast to dexamethasone, is unable itself to induce histone acetylation.

The above data examines bulk histone acetylation status. In order to be functionally relevant these events must occur at the correct promoter sites. Using chromatin immunoprecipitation assays, we showed that H4-K8 acetylation, but not H4-K5 acetylation was involved in IL-1β-stimulated GM-CSF promoter activation. Both dexamethasone and
mifepristone inhibited the IL-1β-stimulated increase of GM-CSF promoter associated with acetylated H4-K8 (Figure 4c). The effect of dexamethasone was greater (64% reduction) than that seen with mifepristone (45% reduction). This data confirms the earlier results showing that dexamethasone was more effective than mifepristone in inhibiting GM-CSF release and also suggests that the effect occurs at the level of gene expression. This was confirmed using RT-PCR. This showed that IL-1β induced expression of both GM-CSF and SLPI steady-state mRNA (Figure 4d, compare lanes 1 and 2). GM-CSF steady state mRNA levels were reduced to a greater extent with dexamethasone (1µM, 95%) than by mifepristone (1µM, 51%)(Figure 4d, compare lanes 5 and 8) mimicking the effect seen at the protein level. SLPI steady-state mRNA expression also followed its release patterns with a reduction at low concentrations of dexamethasone (10⁻⁹M, lane 3) and subsequent gene induction at higher concentrations (10⁻⁶M, lane 5). In contrast, mifepristone reduced SLPI steady-state mRNA levels at low concentrations (10⁻⁹M, lane 7) without subsequent induction of gene expression at higher concentrations (Figure 4d, lane 8). This data indicates that release is controlled at the level of gene expression. Although not formally demonstrated here, previous data has suggested that dexamethasone regulation of GM-CSF release in lung epithelial cells is regulated at the level of gene transcription (30)

**Effect of mifepristone on p65-induced histone acetylation and deacetylation**

In order to clarify the inhibitory mechanism of mifepristone on histone acetylation, we investigated p65-associated histone acetylation and deacetylation in IL-1β and/or mifepristone or dexamethasone stimulated cells. We have previously shown that IL-1β-stimulated HAT activity induced acetylation of K5 and K12 residues only after mild, but not
stringent, CBP-immunoprecipitation conditions suggesting that CBP itself does not play a role in NF-κB-induced histone acetylation (18). Histone acetylation was increased 3-fold following IL-1β stimulation (p<0.05, Figure 5a). Dexamethasone and mifepristone both inhibited p65-associated IL-1β-induced histone acetylation in a concentration-dependent manner (IC\(_{50}\) =3.7 x 10\(^{-10}\)M and 3.1 x 10\(^{-7}\)M, p<0.05) (Figure 5a open bars). Dexamethasone and mifepristone, in the absence of IL-1β, produced no change in p65-associated histone acetylation from that seen in control untreated samples (Figure 5a open bars). In order to confirm that dexamethasone and mifepristone targeted p65-associated HAT activity specifically, control experiments with a blocking peptide were performed, which showed that no histone acetylation activity was pulled down in these assays (184 ± 41 versus >2500dpm/10\(^6\) cells). To confirm the earlier data on GM-CSF release (Figure 1d) we examined the effect of HDAC inhibition on dexamethasone and mifepristone actions on IL-1β-induced p65-associated HAT activity. TSA (10 ng/ml) reduced dexamethasone suppression of p65-associated HAT activity (IC\(_{50}\) =1.9 x 10\(^{-8}\)M versus 3.7 x 10\(^{-10}\)M, p<0.05) but had no effect on the mifepristone concentration-response curve (IC\(_{50}\) =5.5 x 10\(^{-8}\)M versus 3.1 x 10\(^{-7}\)M) (Figure 5a closed bar). In the presence of TSA, there was no significant difference in IC\(_{50}\) for IL-1β-stimulated-p65 associated HAT activity between dexamethasone and mifepristone (IC\(_{50}\) =1.9 x 10\(^{-8}\)M versus 5.5 x 10\(^{-8}\)M).

In the same immunoprecipitates, mifepristone was found to have little or no effect on histone deacetylation except at the highest concentrations studied (10\(^{-6}\)M: 103.2 ± 15.0
versus 15.3 ± 3.9 dpm/\(10^6\) cells, \(p<0.05\)). In comparison, dexamethasone caused a concentration-dependent increase in p65-associated HDAC activity (\(10^{-6}\)M: 939.4 ± 58.3 dpm/\(10^6\) cells versus 15.3 ± 3.9, \(p<0.01\)) (Figure 5b).

**Effect of mifepristone on HDAC expression, activity and recruitment**

We have previously shown that dexamethasone induced HDAC2 recruitment to the p65-associated HAT complex. We therefore examined whether this was the case for mifepristone. We determined the effect of mifepristone on HDAC2 expression, histone deacetylase activity and p65/HDAC association. In marked contrast to dexamethasone, mifepristone failed to induce either HDAC2 expression or histone deacetylation activity (Fig 6a). Western blot analysis of p65-immunoprecipitates showed a recruitment of HDAC2 to the p65 immunoprecipitated complex following treatment of cells with IL-1β and low concentrations (\(10^{-8}\)M) of dexamethasone (Figure 6b), suggesting a role for HDAC2 in the suppressive actions of dexamethasone. In contrast, mifepristone failed to mediate recruitment of HDAC2 to the p65-associated HAT complex.
Discussion

It has been postulated that mifepristone and related compounds may dissociate transrepression from transactivation at AP-1 and NF-κB driven promoters (19;20). Therefore, we used mifepristone to examine the roles of GR transactivation and transrepression in the control of GM-CSF and SLPI expression and histone acetylation status. Mifepristone was unable to stimulate histone H4 acetylation and SLPI release. IL-1β caused a concentration-dependent increase in GM-CSF expression, which was inhibited by 50% maximally by mifepristone. A similar effect of mifepristone was seen on IL-1β-stimulated histone acetylation and on p65-associated HAT activity. We have previously demonstrated that dexamethasone was able to inhibit IL-1β-stimulated histone acetylation by a combination of direct inhibition of p65-activated HAT activity and by recruitment of HDACs to the activated p65/HAT complex. Here we show that mifepristone has a similar ability to dexamethasone in repressing p65-associated HAT activity but, in contrast, is unable to recruit HDAC2 to the activated p65/HAT complex. We have previously shown that this p65-induced HAT activity is associated with, but not due to, CBP and PCAF (18). We show that the p65-associated HAT activity was directly inhibited by both dexamethasone and mifepristone in vitro suggesting that this is the target for glucocorticoid action rather than CBP itself. It is possible that CBP may play a scaffolding role in this process.

Many previous studies have reported a role for CBP in mediating NF-κB activity and or its interactions with GR (31-37). All of these studies involve over-expression of one or more of the factors thought to be involved in the interactions or micro-injection of antibodies. As such these must be considered as contrived systems which must be confirmed in the natural cell. In this study, in the absence of over-expression, we were unable to show any role for CBP, or
PCAF, in mediating the IL-1β-stimulated p65-associated increase in histone acetyltransferase and GM-CSF gene expression. An alternative scenario is that dexamethasone and mifepristone inhibit p65 association with co-activators such as CBP and PCAF. Although not measured directly here we have previously shown that this is not the case for dexamethasone (18).

Many of the anti-inflammatory effects of corticosteroids may be mediated by repression of transcription factors (transrepression), whereas the endocrine and metabolic effects of corticosteroids are mediated via GRE binding (transactivation)(38). This has led to a search for novel corticosteroids that selectively repress inflammatory gene transcription and would thus reduce the risk of systemic side effects. Transactivation via GR/GRE binding involves a GR homodimer, whilst transrepression of transcription factor (AP-1 and NF-κB) activity involves a GR monomer. A separation of transactivation and transrepression has been demonstrated using reporter gene constructs in transfected cells using selective mutations of GR (19). Furthermore, some corticosteroids, such as RU24858, mifepristone and ZK98299, have a greater transrepression than transactivation effect (19;20). Indeed, the topical corticosteroids used in asthma therapy today, such as fluticasone propionate and budesonide, appear to have more potent transrepression than transactivation effects, which may account for their selection as potent anti-inflammatory agents (39). Recently, a novel class of corticosteroids has been described in which there is potent transrepression with relatively little transactivation. These “dissociated” corticosteroids, including RU24858 and RU40066 have anti-inflammatory effects in vivo (21).

The clinical relevance of these effects of GR mutants is indicated by the construction of a GR dimerisation-deficient mutant mouse in which GR is unable to dimerise and therefore
bind to DNA, thus separating the transactivation and transrepression activities of corticosteroids (40). These animals, in contrast to GR knockout animals, survive to adulthood. In these animals dexamethasone was able to inhibit AP-1-driven gene transcription but the ability to facilitate GRE-mediated effects such as cortisol suppression and T-cell apoptosis were markedly inhibited. This suggests that the development of corticosteroids with a greater margin of safety is possible and may predict the development of oral corticosteroids that may be safe to use in asthma and other inflammatory diseases. The results of glucocorticoid actions on airway hyperresponsiveness and airway inflammation in these animals are waiting to be determined.

The results presented in this study differ from those of Heck and colleagues (20) in regards to the ability of mifepristone to cause transrepression and others with regards to NF-κB requirement for CBP (31). The most obvious reason for this is the use of reporter gene assays and GR over-expression in the study of Heck whilst they were not employed here. This has important ramifications for the use of reporter genes and over-expression assays in understanding the role of specific proteins in complex systems.

In summary, we have shown that the glucocorticoid receptor agonist mifepristone has no ability to induce gene transcription but represses IL-1β-stimulated histone acetylation and GM-CSF release by 50% maximally. IL-1β-stimulated NF-κB activated distinct p65-associated HATs (35 and 55kDa) but did not activate CBP or PCAF HAT activity. Mifepristone was able to inhibit p65-associated HAT activity to the same extent as dexamethasone but failed to recruit HDAC2 to the p65/HAT complex. This data suggests that the maximal transcriptional repressive action of glucocorticoids requires recruitment of HDAC2 to the p65/HAT complex. In addition, our results suggest dissociation between the
ability of mifepristone to repress histone acetylation and gene expression and with its ability to activate histone acetylation and to switch on gene expression. This suggests that our model of histone acetylation/deacetylation may prove to be useful for the examination of dissociated glucocorticoids.
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Figure legends.

Figure 1. Mifepristone inhibits IL-1β-stimulated gene expression.

(a) Inhibitory effects of mifepristone and dexamethasone on IL-1β↑-induced GM-CSF production. Cells were pre-incubated with mifepristone (10^{-12} - 10^{-5} M) or dexamethasone (10^{-12} - 10^{-6} M) for 30 min before incubation with IL-1β (1 ng/ml) for 24 hours. Supernatants were collected and assayed for GM-CSF by ELISA. Results are expressed as mean ± SEM, n= at least 3 independent experiments, *p<0.05, **p<0.01. NS = non-stimulated.

(b) Effects of mifepristone and dexamethasone on SLPI production. Cells were incubated for 24 hours before supernatants were collected and assayed for SLPI by ELISA. Results are expressed as mean ± SEM. Each experiment was performed in duplicate on 3 separate occasions, **p<0.01.

(c) Effect of mifepristone and dexamethasone on IL-1β-stimulated SLPI production. Cells were pre-incubated with concentrations of mifepristone or dexamethasone for 30 min before incubation with IL-1β (1 ng/ml) for 24 hours. Supernatants were collected and assayed for SLPI release by ELISA. Results are expressed as mean ± SEM, n= at least 3 independent experiments, *p<0.05 **p<0.01.

(d) Effect of TSA on the inhibitory effect of mifepristone (Mif) and dexamethasone (Dex) on IL-1β↑-induced GM-CSF production. Cells were pre-incubated with concentrations of Mif (10^{-6} M) or Dex (10^{-6} M) for 30 min before incubation with IL-1β (1 ng/ml) for 24 hours. Supernatants were collected and assayed for GM-CSF by ELISA. T(e) SA (10 ng/ml) was added 10 min before IL-1β-stimulation. Results are expressed as mean ± SEM, n= at least 3 independent experiments, *p<0.05, **p<0.01 versus IL-1β alone control, #p<0.05.
versus TSA treatment.

**Figure 2. Effect of mifepristone on IL-1β-induced histone acetylation.**

Mifepristone inhibits IL-1β-induced histone acetylation in total cell extracts. Cells were pretreated with mifepristone or dexamethasone for 30 min before incubation with IL-1β (1 ng/ml) for 6 hrs in the presence of 0.05 mCi [3H]-acetate. Histones were isolated and separated by SDS-PAGE and [3H]-acetate incorporated histones were counted and normalised to protein level. Data represents mean ± SEM of 3 independent experiments. **p<0.01, *p<0.05.

**Figure 3. Immunocytochemistry of glucocorticoid receptor (GR), NF-κB (p65 subunit) and acetylated histone 4.**

Cells were incubated with Mifepristone (10⁻⁶ M) (b, f, j & n), Dexamethasone (10⁻⁶ M) (c, g, k & o) or IL-1β (1 ng/ml) (d, h, l & p) for 6 hrs. Cells were analysed for nuclear localisation of GR (a-d), NF-κB (p65 subunit) (e-h), acetylated lysine residues K5 (i-l) and K8 (m-p) by immunocytochemistry. Results are representative of 4 independent experiments.

**Figure 4. Effect of mifepristone on IL-1β-induced specific histone acetylation and promoter activation.**

A. Cells were incubated with IL-1β (1 ng/ml) for 6 hrs in the presence of mifepristone (Mif, 10⁻⁶ M) or dexamethasone (Dex, 10⁻⁶ M). Protein extracts were obtained and examined for acetylated histone H4 lysine residue K5 by Western blot analysis. Results are representative of 3 independent experiments. Densitometric analysis was also done and
results presented as mean ± SEM of at least 3 independent experiments. *p<0.05, **p<0.01.

B. Western blot analysis of acetylated histone H4 lysine residue K8 in the same samples as above. Results are representative of 3 independent experiments. Densitometric analysis was also done and results presented as mean ± SEM of at least 3 independent experiments. *p<0.05.

C. Association of acetylated histone 4 K5 and K8 with the GM-CSF promoter. A549 cells pre-treated with mifepristone (Mif, 10^{-6}M) or dexamethasone (Dex, 10^{-6}M) for 30 minutes were incubated with IL-1β (1ng/ml) for 4 hours. Proteins and DNA were cross-linked by formaldehyde treatment and chromatin pellets extracted. Following sonication, acetylated histone H4 K5 and K8 were immunoprecipitated and the associated DNA amplified by PCR. Results are representative of 3 independent experiments.

D. GM-CSF and SLPI are regulated at the level of gene expression. IL-1β (1ng/ml, lane 2) stimulates the expression of both GM-CSF and SLPI mRNA compared with unstimulated cells (lane 1) at 6 hours as measured by RT-PCR. The effect of increasing concentrations of dexamethasone (Dex, lanes 3-5) and mifepristone (Mif, lanes 6-8) on IL-1β-stimulated GM-CSF and SLPI steady-state mRNA expression are shown. GAPDH expression is used as a control for mRNA extraction. Results are representative of 3 independent experiments.

**Figure 5. p65-associated histone acetylation and deacetylation**

(A) EFFECTS OF MIFEPRISTONE (MIF) AND DEXAMETHASONE (DEX) on IL-1β-induced p65-immunoprecipitated histone acetylation in the absence (open bar) or presence (closed bar)
of trichostatin A (TSA, 10 ng/ml). Cells were pre-incubated with increasing concentrations
of Mif or Dex for 30 min before IL-1β (1ng/ml) treatment for (B) a further 1 hr. Total cellular
proteins were isolated and p65 immunoprecipitated. The associated histone acetylation
activity was measured following incubation of the p65-IP extract with 10µg free core
histones and 0.25μCi of ³H-acetyl CoA for 45 minutes. Radiolabelled histones were
counted and results presented as mean ± SEM of at least 3 independent experiments.

*p<0.05 compared to non-stimulated cells. **p<0.05 compared to IL-1β-stimulated cells.

(C) Effects of Mifepristone and dexamethasone on p65-associated histone deacetylation.
Using the same immunoprecipitates as in (A) histone deacetylase activity was measured
by incubation of extracts with ³H-labelled histones for 30mins. Free ³H-labelled acetic
acid was extracted by ethylacetate and measured by liquid scintillation counting. Results
are presented as mean ± SEM of at least 3 independent experiments. **p<0.01.

Figure 6. Effect of Mifepristone and dexamethasone on HDAC protein expression, HDAC
activity and HDAC recruitment to the p65 complex.

(A) Effect of mifepristone and dexamethasone on HDAC2 protein expression and HDAC
activity. Cells were incubated increasing concentrations of mifepristone (10^-8 ,10^-6 M) for
24hrs. Western blot analysis of HDAC2 expression is shown (upper panel) and total
cellular HDAC activity shown in the lower panel. Results are expressed as mean ± SEM
of 3 separate experiments. *p<0.05, p<0.01.

(B) Recruitment of HDAC2 to p65 immunoprecipitated complexes. Cells were incubated with
IL-1β in the presence mifepristone (Mif, 10⁻⁸, 10⁻⁶ M) or dexamethasone (Dex, 10⁻⁸, 10⁻⁶ M) for 4 hrs. Total cellular proteins were isolated and immunoprecipitated with anti-p65 antibodies. HDAC2 content in the immunoprecipitated complexes was measured by Western blotting. The level of p65 within the same samples is shown as a control for protein loading. The result is representative of 4 separate experiments. NS = non-stimulated.
Ito et al., Fig. 2

**Mifepristone**

**Histone acetylation (dmp/μg protein)**

- Log [steroid] (M)

- Non-stim

- IL-1β

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**Dexamethasone**

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Ito et al., Fig. 3

GR

NF-κB:
p65

AcH4K5

Acl14K8
Ito et al., Fig. 6

A

Band density (versus β-actin)

|        | NT       | Mifepristone 8 | Mifepristone 6 | Dexamethasone 8 | Dexamethasone 6 |
|--------|----------|----------------|----------------|-----------------|-----------------|
| Density| 0.00     | 0.25           | 0.50           | 0.75            | *               |

B

- α HDAC2
- α p65

| Treatment | NS | MIF | Dex | IL-1β |
|-----------|----|-----|-----|-------|

HDAC activity (c.p.m./μg protein)

|        | NT | Mifepristone 8 | Mifepristone 6 | Dexamethasone 8 | Dexamethasone 6 |
|--------|----|----------------|----------------|-----------------|-----------------|
| Activity| 0  | 50             | 100            | 150             | *               |
p65-activated histone acetyltransferase activity is repressed by glucocorticoids: Mifepristone fails to recruit HDAC2 to the p65/HAT complex
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