Glucocorticoid suppression of nuclear factor-κB: a role for histone modifications

M. Kagoshima, K. Ito, B. Cosio and I.M. Adcock
Thoracic Medicine, National Heart & Lung Institute, Imperial College School of Medicine, Dovehouse Street, London SW3 6LY, U.K.

Abstract
Corticosteroids are by far the most effective treatment for chronic inflammatory diseases such as asthma. Inflammation in asthma is characterized by the increased expression of multiple inflammatory genes, including those that encode cytokines, chemokines, adhesion molecules, and inflammatory enzymes and receptors. Increased expression of inflammatory genes is regulated by pro-inflammatory transcription factors, such as nuclear factor-κB (NF-κB). These bind to, and activate, co-activator molecules that then acetylate core histones resulting in elevated gene transcription. Corticosteroids reverse histone acetylation at the site of inflammatory gene transcription, either by direct binding of the activated glucocorticoid receptor to NF-κB-associated co-activators or by recruitment of histone deacetylases to the activated transcription complex. Understanding how corticosteroids work in asthma may help in designing novel corticosteroids with fewer systemic effects, as well as novel anti-inflammatory approaches.

Introduction
Inflammation is a central feature of many lung diseases, including asthma [1]. The inflammatory response involves the recruitment to, and activation of, inflammatory cells and changes in the structural cells of the airway. This is thought to involve a complex cascade of inflammatory mediators whose expression is enhanced during the disease process [1]. Since many of these inflammatory genes are not expressed under resting conditions, the increased expression of these proteins must result from cell-specific gene transcription [1]. Glucocorticoids are the most effective therapy in the long-term control of asthma and they appear to reduce inflammation in asthmatic airways, largely by inhibiting abnormal gene expression [2]. Although it is not yet possible to be certain of the most critical aspects of glucocorticoid action in suppressing inflammation, it is likely that their inhibitory effects on cytokine and chemokine synthesis are particularly important. Glucocorticoids inhibit the transcription of several cytokines and chemokines that are relevant in inflammatory lung diseases, including tumour necrosis factor (TNF)-α, granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1β, IL-4, IL-5, IL-8 and eosinotxin [2]. Glucocorticoids may not only block the synthesis of cytokines, but may also block their effects by inhibiting the synthesis of cytokine receptors, such as the IL-2 receptor [2].

In order to appreciate how glucocorticoids function in modifying inflammatory mediator release, it is important to understand how the expression of these mediators is regulated.

Nuclear factor-κB (NF-κB)
Although numerous different pathways are activated during the inflammatory response, one particular type of pathway is thought to be of paramount importance in inflammation and immunology, i.e. a pathway that involves NF-κB [3]. This was first described in B-cells, but is now recognized as one of the most important immunoregulatory genes in mammalian cells [4]. NF-κB is activated by all the stimuli that are thought to be important in the inflammatory response to allergen exposure seen in asthma and is the major target for glucocorticoids [5]. NF-κB is expressed ubiquitously within cells and can both control the induction of inflammatory genes in its own right, and modify and enhance the activity of cells and signal-specific transcription factors, such as nuclear factor of activated T-cells (NFAT), [6] and signal specific activators, such as signal transducers and activators of transcription (STAT) [7].

NF-κB is activated by extracellular stimuli including cytokines, such as TNF-α and IL-1β, viruses and immune challenges [4]. Activation of cell surface receptors leads to phosphorylation of receptor-associated kinases [5]. These kinases phosphorylate a specific intracellular kinase inhibitor of NF-κB kinase kinase. Phosphorylation of this kinase results in phosphorylation of the cytoplasmic inhibitor of NF-κB (IkBα), which targets the IkBα for proteosomal degradation. This releases NF-κB from its inactive state enabling nuclear translocation and binding to specific DNA

Key words: acetylation, asthma, epithelial cell, granulocyte/macrophage colony-stimulating factor (G-CSF), inflammation.

Abbreviations used: AP-1, activating protein-1; CBP, CAMP-response-element-binding protein; HDAC, histone deacetylase; hsp90, heat-shock protein of 90 kDa; IL, interleukin; IL-1R, IL-1 receptor; iκBα, inhibitor of NF-κB; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein-1; NF-κB, nuclear factor-κB; PCAF, p300/CBP-associated protein; RANSES, regulated upon activation, normal T-cell expressed and secreted; TNF, tumour necrosis factor.

To whom correspondence should be addressed (e-mail ian.adcock@ic.ac.uk).
response elements within the promoter regions of responsive genes [5].

NF-κB DNA-binding activity in cells such as macrophages from induced sputum and in biopsies from mild asthmatic patients is increased, and the expression of this transcription factor was increased in the airway epithelium of patients with mild asthma [8]. The airway epithelium in asthma is the site of enhanced expression of several NF-κB-inducible pro-inflammatory proteins including cytokines such as GM-CSF, RANTES (regulated upon activation, normal T-cell expressed and secreted) and monocyte chemotactic protein-1 (MCP-1), enzymes such as inducible nitric oxide synthase and cyclo-oxygenase 2 (COX-2), and adhesion molecules such as intercellular adhesion molecule-1 [9].

**Histone modifications and gene transcription**

**Histone acetylation**

At a microscopic level, it has long been recognized that chromatin may become dense or translucent owing to the winding or unwinding of DNA around core histones [10]. Acetylation of histone residues results in unwinding of the DNA that is wrapped around the histone core. This process opens up the chromatin structure, allowing transcription factors and RNA polymerase II to bind more readily to DNA, and thereby increasing gene transcription [11].

Transcriptional co-activators such as cAMP-response-element-binding protein-binding protein (CBP) and p300/CBP-associated protein (PCAF) have intrinsic histone acetyltransferase (HAT) activity, which is activated by the binding of transcription factors [11,12]. Increased gene transcription is therefore associated with an increase in histone acetylation, whereas hypo-acetylation is correlated with reduced transcription or gene silencing [11]. Histone acetylation is an active process whereby small changes in acetylas in histone deacetylases (HDACs) can markedly affect the overall histone acetylase activity associated with inflammatory genes [11,13]. Importantly, these changes in histone acetylation are targeted to regions of DNA associated with specific activator sites within the promoters of induced inflammatory genes [11], although a global loosening of histone structure has also been proposed [14].

Specific residues (lysine, arginine and serine) within the N-terminal tails are capable of being post-translationally modified, by acetylation, methylation or phosphorylation [15]. Acetylation of the σ-group on lysine residues reduces the charge of the histone residue and subsequently releases the tightly wound DNA, inducing a relaxed DNA structure that allows the recruitment of further large protein complexes [11].

**Histone deacetylation**

Repression of genes is associated with reversal of this process by histone deacetylation, a process controlled by HDACs [13]. HDACs are part of growing family of enzymes of which at least 10 mammalian forms exist [16]. Two distinct classes of HDACs have been identified in mammalian cells. Class I members, such as HDAC1, HDAC2, HDAC3 and HDAC8, are transcriptional co-repressors that are homologous to yeast Rpd3. Class II members, including HDAC4, HDAC5, HDAC6, HDAC7 and HDAC9, possess domains similar to the deacetylase domain of yeast Hdal. HDAC4, HDAC5 and HDAC7 can interact with the Mads box enhancer factor 2 (MEF2) transcription factors and the nuclear receptor co-repressor (N-CoR), Bel-6 interacting co-repressor (BcoR) and C-terminal binding proteins (CtBP) co-repressors [17]. Interestingly, HDAC4, HDAC5, and probably HDAC7, are regulated through subcellular compartmentalization controlled by site-specific phosphorylation and binding of 14-3-3 proteins; the regulation of these HDACs is thus directly linked to cellular signalling networks [16,17].

Deacetylation of histones increases the winding of DNA around histone residues, resulting in a dense chromatin structure and reduced access of transcription factors to their binding sites, thereby leading to repressed transcription of inflammatory genes [13].

**NF-κB induces histone acetylation**

By activating NF-κB, IL-1β can induce histone acetylation in both a time- and concentration-dependent manner in lung epithelial cells (A549 cells) [18]. This NF-κB-induced acetylation occurs preferentially on histone H4, rather than histones 2A, 2B or 3, and is directed towards Lys4 and Lys12. In addition, the acetylation of Lys8 and Lys12 occurs at promoters that are NF-κB-inducible and are dependent upon binding of NF-κB to DNA [18]. Upon DNA binding, NF-κB recruits a large co-activator complex that contains the HAT proteins, CBP and PCAF [18]. The overall HAT activity of this NF-κB-associated complex is enhanced, possibly owing to phosphorylation of the individual HATs that are recruited to this complex [19–21]. Several other HATs have been reported to be associated with NF-κB and include transcriptional intermediary factor 2 (TIF-2) [22] and p300 [23].

**Temporal association of NF-κB with DNA, cofactors and gene induction**

The initial, simplistic view of NF-κB activation required its interaction with DNA immediately following its activation and that it remained DNA-bound throughout gene transcription. However, in a series of beautiful studies using chromatin immunoprecipitation assays, Saccani et al. [24,25] have shown that this model needs modification. NF-κB binds rapidly to the promoters of immediate early genes, such as that of IκBα, after lipopolysaccharide stimulation, but within 10 min, NF-κB dissociates from the IκBα promoter site and never re-associates. In contrast, NF-κB was found to bind to its promoter sites in DNA for up to 2 h before dissociation in distinct sets of genes (manganese superoxide dismutase and macrophage inflammatory protein-2) in spite of stimulation.
by lipopolysaccharide at the same time. However, NF-κB did not bind to the promoters of other NF-κB-regulated genes, such as RANTES, MCP-1 and IL-6, until 2 h after activation. In these elegant studies, it was reported that the NF-κB sites in the promoter regions of these genes were originally in a repressed chromatin environment, which prevented binding of NF-κB to DNA, and only became accessible after activating protein-1 (AP-1)-mediated histone acetylation and subsequent alteration in the local nucleosomal structure [24,25]. Thus, there are subtle changes in NF-κB DNA binding that are promoter context-dependent, that precede co-activator recruitment and that are not detected using conventional band shift and reporter gene assays. Similar time-dependent recruitment of factors at specific promoters has also been reported elsewhere [26].

Regulation of NF-κB activity by trichostatin A

The HDAC inhibitor trichostatin A has been reported to enhance NF-κB-driven inflammatory gene transcription in a number of cell lines [18,27,28]. Two major mechanisms for this effect have been proposed. In the first case, it has been reported that NF-κB has an associated HDAC when bound to DNA, which prevents NF-κB from activating local HAT activity. Inhibition of this associated HDAC leads to increased local HAT activity and elevated inflammatory gene transcription [18,27]. An alternative mechanism has been proposed by Greene and colleagues [28] who showed that HDAC3 can modify NF-κB nuclear-cytoplasmic shuttling and association with IκB. This results in enhanced nuclear retention of activated NF-κB, which is insensitive to inactivation by IκB. These results suggest that HDAC activity is important in the regulation of inflammatory gene expression and further evidence for this is seen in human broncho-alveolar lavage macrophages, where TNF-α-induced GM-CSF release is inversely correlated with HDAC activity [29]. Regulation of p65 activity by acetylation is yet another example of how acetylation of transcription factors by recruited co-activator molecules can modify transcription factor activity [30–33].

Glucocorticoid receptor (GR)

Glucocorticoids exert their effects by binding to the cytoplasmic GRs [34]. GRs are expressed in almost all cell types and are modular in structure. Thus, GR has several functional domains including a ligand-binding domain, a DNA-binding domain and two domains that are involved in transactivation of genes once binding to DNA has occurred, via association with other proteins [34]. The inactive GR is bound to a protein complex that includes two subunits of the heat-shock protein of 90 kDa (hsp90), which thus act as molecular chaperones preventing the nuclear localization of unoccupied GR [34]. Once the ligand binds to GR, hsp90 dissociates allowing the nuclear localization of the activated GR–steroid complex and its binding to specific DNA sequences (glucocorticoid response elements, or GREs; GGTACanntGTGTTCT) or interaction with co-activator complexes [34].

GR, like other steroid hormone receptors, is a phospho-protein, and changes in their phosphorylation status may modulate their activity. Several of these sites lie in consensuses for proline-directed, cell-cycle-associated kinases and mitogen-activated protein kinases. Receptor phosphorylation may influence the interactions of GR with other transcription factors that are required for transactivation. Phosphorylation of the unoccupied GR on a tyrosine residue in the ligand-binding domain may block subsequent hormone binding and nuclear translocation [35]. In addition, phosphorylation of the rat GR by c-Jun N-terminal kinase (JNK) directly, and by extracellular signal-regulated protein kinases indirectly, have been reported to inhibit GR-dependent gene transcription [36].

Induction of gene transcription

Binding of the GR homodimer to GREs changes the rate of transcription, resulting in the induction of steroid-responsive genes. Other transcription factors binding in the vicinity of a GRE may also have a powerful influence on glucocorticoid inducibility and the relative abundance of different transcription factors may contribute to the responsiveness of a particular cell type. GR–DNA interactions changes DNase1 sensitivity, indicating that there may be a local change in DNA or chromatin configuration, which may expose previously masked areas, resulting in increased binding of other transcription factors and the formation of a more stable transcription initiation complex [34].

Glucocorticoids increase the synthesis of anti-inflammatory proteins, including lipocortin-1, serum leukoprotease inhibitor, Clara Cell protein 10 and IL-1 receptor (IL-1R) antagonist, and these effects are presumably mediated via GREs in the promoter regions of these genes [2]. Glucocorticoids have also been reported to increase the expression of IκB in lymphocytes and thereby to inhibit NF-κB, but this has not been seen in other cell types [2].

IL-1β acts at two types of receptor designated IL-1RI and IL-1RII. The inflammatory effects of IL-1β are mediated exclusively via IL-1RI, whereas IL-1RII has no signalling activity, but binds IL-1β and therefore acts as a molecular trap that interferes with the actions of IL-1β. Glucocorticoids are potent inducers of the decoy IL-1RII and result in the release of a soluble form of the receptor, thus reducing the functional activity of IL-1β [2].

Although they are rare, a few true negative GREs have been reported of which the most interesting is that of the osteocalcin gene. Osteocalcin is repressed by GR acting through a GRE that overlaps the TATA box, thereby blocking the binding of the basal transcription complex and therefore mRNA expression [37].

GRs, as with other transcription factors, increase gene transcription through an action on chromatin remodelling
and recruitment of RNA polymerase II to the site of local DNA unwinding, as described above for pro-inflammatory transcription factors. GR interacts with CBP and other co-activator proteins, including steroid receptor coactivator-1 (SRC-1), TIF-2, p300/CBP co-integrator protein (p/CIP) and glucocorticoid receptor interacting protein (GRIP)-1, which enhance local HAT activity [38]. High concentrations of dexamethasone (≥10^{-4}M) in A549 cells result in binding of activated GR to CBP and/or associated co-activators, which results in histone acetylation on Lys^8 and Lys^{12} of histone H4 and increased gene transcription [18].

### Cross-talk between GR and other transcription factors

Despite the ability of glucocorticoids to induce gene transcription, their major anti-inflammatory effects are through repression of inflammatory and immune genes. The inhibitory effect of glucocorticoids appears to be due largely to a protein–protein interaction between activated GR and transcription factors, such as NF-κB and AP-1, which mediate the expression of these inflammatory genes [39]. Direct protein–protein interactions have been demonstrated between GR and AP-1, and between the p65 component of NF-κB and GR, which suggests that glucocorticoids modulate either the binding or activation of these transcription factors and thus modify the expression of inflammatory genes [39].

The interplay between pro-inflammatory transcription factors and GR may reflect differing effects on histone acetylation/deacetylation. This may occur through one of several mechanisms that are probably not exclusive. The repressive action of glucocorticosteroids may be due to competition between GR and the binding sites on CBP for other transcription factors, including AP-1 and NF-κB. Alternatively, activated GR may bind to one of several transcription co-repressor molecules, such as the receptors receptor interacting protein 140 (RIP140) and NcoR1, which associate with proteins that have differing HDAC activity [38]. Thus, IL-1β and TNF-α can cause histone acetylation of Lys^{8} and Lys^{12} of histone H4 and low concentrations of dexamethasone (≥10^{-9}M) can repress this IL-1β-stimulated histone acetylation. This occurs by a direct inhibition of CBP-associated HAT activity and by active recruitment of HDAC proteins [18]. In addition, high concentrations of glucocorticoids can induce HDAC expression in a time-dependent manner [18]. Overall, this results in the deacetylation of histones, increased tightening of DNA around histone residues, and the repression of inflammatory genes [18]. According to the histone code [40], other histone modifications would be expected to play a role in GR/NF-κB cross-talk at the level of chromatin. We have recently shown this to be the case for histone H3 methylation, which acts together with H4 acetylation to modify GR function [41].

Competition between pro- and anti-inflammatory transcription factors for limited amounts of cofactors, such as CBP, may occur resulting in a reduction in the expression of inflammatory genes. Full inflammatory gene expression probably requires a number of transcription factors acting together in a co-ordinate manner, and repression of a single transcription factor alone may only partially modify the full response. Glucocorticoids by repressing a down-stream target of transcription factor activation, may be able to reduce inflammatory gene expression irrespective of the precise activated transcription factors involved. Recent results have suggested a further mechanism for GR action. The phosphorylation of RNA polymerase II serine residues, induced by NF-κB at the IL-8 promoter, is reduced by GR without affecting the assembly of the pre-initiation complex. This suggests an action for GR downstream of NF-κB–DNA binding [42]. Other models propose that GR interferes with p65 association with the TATA box environment thus inhibiting p65 actions (Figure 1) [43].

GR has also been shown to prevent c-Jun phosphorylation on Ser^{32}/Ser^{39} and, subsequently, AP-1 activation, by blocking the induction of the JNK signalling cascade. Consistent with this, nuclear receptors also antagonize other JNK-activated transcription factors, such as Elk-1 and activating transcription factor 2 [44]. Interference with the JNK signalling pathway represents a novel mechanism, by which nuclear hormone receptors antagonize AP-1.

The importance of cross-talk in GR actions is indicated by the construction of a GR dimerization-deficient mutant mouse in which GR is unable to dimerize and therefore bind to DNA, thus separating the transactivation and transrepression activities of glucocorticoids [45,46]. These animals, in contrast with 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 glucocorticoids with a greater therapeutic window is possible.

### Glucocorticoid resistance

A small proportion of asthmatic patients are glucocorticoid-resistant and fail to respond to even high doses of oral corticosteroids [47]. Similar resistance is reported in other chronic inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis. Monocytes and T-lymphocytes isolated from these patients have an impaired response to glucocorticoids in vitro. In some patients, there is a reduction in the affinity of GR for glucocorticoids and this can be mimicked by incubation of T-cells with IL-2 and IL-4, which leads to a functional inhibition of glucocorticoid action [48].

There is also a marked reduction in the number of activated GRs within the nucleus after exposure of mononuclear cells to glucocorticoids in vitro, compared with cells from normal individuals and glucocorticoid-sensitive asthmatics [48]. In the same patients, there is a reduced inhibitory effect of glucocorticoids on AP-1 activation and cytokine expression,
Figure 1 | NF-κB recruitment of HATs, formation of PIC and inhibition by glucocorticoids

Activation of NF-κB by cytokines such as IL-1β leads to DNA binding and recruitment of HATs such as CBP/p300 and PCAF to the NF-κB site in the promoter of inducible genes. This results in acetylation of histone residues within the nucleosomes close to NF-κB, and a local decrease in charge allows DNA unwinding to occur and further recruitment of large protein complexes including the TATA-binding protein (TBP)-associated factor (TAF250) and RNA polymerase II (RNA pol II). This is then associated with enhanced gene transcription. Phosphorylation of CBP by IL-1β-induced kinases may also enhance binding of NF-κB to DNA and transcriptional activity. Glucocorticoids may also enhance gene expression through a similar process. Activation of GR by ligands results in binding to specific DNA sites in responsive genes (GREs) and recruitment of cofactors similar to those used by NF-κB to induce chromatin modifications and stimulate GR-inducible gene expression. Alternatively, GR may repress NF-κB-mediated gene induction by interfering with the ability of NF-κB to stimulate chromatin modifications either by a direct effect on CBP-associated HAT activity or by recruitment of HDACs. Recruitment and activation of RNA pol II may also be a target for GR actions. Ac, acetyl; SLPI, serum leukoprotease inhibitor.

Possibly owing to enhanced activation of AP-1 and JNK [49,50]. The increased activation of AP-1 may result in sequestration of GR, thereby preventing its interaction with other proteins, thus resulting in glucocorticoid resistance. This resistance will be seen at the site of inflammation where cytokines are produced, i.e. in the airways of asthmatic patients, but not at non-inflamed sites. This may explain why patients with glucocorticoid-resistant asthma are not resistant to the endocrine and metabolic effects of glucocorticoids, and thus why they develop side effects [47].

In a majority of asthmatic subjects, there is a direct correlation between the ability of GR to translocate into the nucleus of peripheral blood mononuclear cells, for histone residues to be acetylated and for gene transcription to occur [51]. In a smaller subgroup of these more severe patients although the GR translocates into the nucleus, there is a reduced ability to cause histone acetylation. Using acetylated histone-specific antibodies, this defect was localized to a specific residue, Lys5, in histone H4. This residue is a major target for normal glucocorticoid actions, such as the induction of serum leukoprotease inhibitor and T-cell apoptosis. These results suggest that within a small sub-group of glucocorticoid-resistant subjects, there is a defect in GR interactions with the basal transcriptional machinery.

Therapeutic implications

The increased understanding of how glucocorticoids act has given new insights into the pathophysiology of inflammatory diseases, such as asthma, but has also opened an opportunity for the development of new anti-inflammatory treatments. Several new therapies, based on interacting with glucocorticoid-responsive proteins or their activation pathways, are now in development for the treatment of chronic inflammatory diseases (mitogen-activated protein kinase inhibitors, NF-κB inhibitors and histone acetylase inhibitors). One concern about this approach is the specificity of such drugs, but it is clear that these glucocorticoid-responsive pathways have selective effects on the expression of certain genes and this may make it possible to be
more selective. This work has also led to the search for dissociated glucocorticoids, i.e. ligands that can induce transpression but have little or no transactivation in vivo. Another of the most important implications of research on glucocorticoid actions is that multiple and complex interactions between proteins are possible, and that this leads to cross-talk between different signal transduction pathways. This might be exploited therapeutically by the combination of drugs that act on different transcription factors or pathways that may work together co-operatively. As such, we have demonstrated recently that theophylline, by directly increasing HDAC activity, can enhance glucocorticoid responsiveness in epithelial cells and macrophages [52]. In addition, there are cell-specific effects of glucocorticoids, such as T-cell apoptosis, that may be targeted for inhibition, which could provide selectivity of drug action.

This work was funded by research grants from the Clinical Research Committee (Royal Brompton Hospital, London, U.K.), the British Lung Foundation and GlaxoSmithKline.

References


Received 8 October 2002