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SERIES "SIGNALLING AND TRANSCRIPTIONAL REGULATION IN INFLAMMATORY AND IMMUNE CELLS: IMPORTANCE IN LUNG BIOLOGY AND DISEASE"
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Number 4 in this Series

Corticosteroid effects on cell signalling

P.J. Barnes

ABSTRACT: Corticosteroids are the most effective anti-inflammatory therapy for asthma. Inflammation in asthma is characterised by the increased expression of multiple inflammatory genes regulated by pro-inflammatory transcription factors, such as nuclear factor-κB and activator protein-1, which bind to and activate coactivator molecules that acetylate core histones and switch on gene transcription. Corticosteroids suppress the multiple inflammatory genes that are activated in asthmatic airways, mainly by reversing histone acetylation of activated inflammatory genes through binding of glucocorticoid receptors to coactivators and recruitment of histone deacetylase 2 to the activated transcription complex.

Activated glucocorticoid receptors also bind to recognition sites in the promoters of certain genes in order to activate their transcription, resulting in secretion of anti-inflammatory proteins, such as mitogen-activated protein kinase phosphatase-1, which inhibits mitogen-activated protein kinase signalling pathways. Glucocorticoid receptors may also interact with other recognition sites to inhibit transcription, for example of several genes linked to their side-effects.

In some patients with steroid-resistant asthma, there are abnormalities in glucocorticoid receptor signalling pathways. In chronic obstructive pulmonary disease patients and asthmatic patients who smoke, histone deacetylase 2 is markedly impaired as a result of oxidative/nitrative stress, and so inflammation is resistant to the anti-inflammatory effects of corticosteroids.

The therapeutic implications of these new findings are discussed.

KEYWORDS: Glucocorticoid receptor, glucocorticosteroid, steroid resistance, transcription factor

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Corticosteroids (also known as glucocorticosteroids, glucocorticoids or simply steroids) are by far the most effective anti-inflammatory treatment for asthma and have now become the first-line therapy in all patients with persistent asthma and with a number of other inflammatory and immune diseases. There have been important advances in understanding the molecular mechanisms whereby corticosteroids suppress inflammation so effectively in asthma, based on recent developments in understanding the fundamental mechanisms of gene transcription and cell signalling in inflammation [1, 2]. This new understanding of these molecular mechanisms also helps to explain how corticosteroids are able to switch off multiple inflammatory pathways, yet remain a safe treatment. It also provides insights into why corticosteroids fail to work in patients with steroid-resistant asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis [3].

The predominant effect of corticosteroids is to switch off multiple inflammatory genes (encoding cytokines, chemokines, adhesion molecules, inflammatory enzymes, receptors and proteins) that have been activated during the inflammatory process. They have additional effects on the synthesis of anti-inflammatory proteins, and also post-genomic effects. There has been considerable interest in how corticosteroids affect the signal transduction pathways that are activated by inflammation.

Previous articles in this series: No. 1: Fan J, Heller NM, Gorospe M, Atasoy U, Stellato C. The role of post-transcriptional regulation in chemokine gene expression in inflammation and allergy. *Eur Respir J* 2005; 26: 933–947. **No. 2:** Georas SN, Guo J, De Fanis U, Casolaro V. T-helper cell type-2 regulation in allergic disease. *Eur Respir J* 2005; 26: 1119–1137. **No. 3:** Boxall C, Holgate ST, Davies DE. The contribution of transforming growth factor-β and epidermal growth factor signalling to airway remodelling in chronic asthma. *Eur Respir J* 2006; 27: 208–229.

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THE MOLECULAR BASIS OF INFLAMMATION

Chronic inflammatory diseases, such as asthma and COPD, involve the infiltration and activation of many inflammatory and immune cells, which release multiple inflammatory mediators that interact and activate structural cells at the site of inflammation. The pattern of inflammation clearly differs between these diseases, with the involvement of different cells and mediators [4, 5], but all are characterised by increased expression of multiple inflammatory genes, some of which are common to all inflammatory diseases, whereas others are more specific to a particular disease. These inflammatory genes are controlled by pro-inflammatory transcription factors, such as nuclear factor (NF)-κB and activator protein (AP)-1, which become activated during the inflammatory process. These pro-inflammatory transcription factors are activated in all inflammatory diseases and play a critical role in amplifying and perpetuating the inflammatory process. Thus, NF-κB is activated in the airways of asthmatic patients and COPD patients [6, 7]. More disease-specific proteins are more likely to be regulated by cell-specific transcription factors, such as nuclear factor of activated T-cells, which regulates certain cytokine genes in T-lymphocytes [8], or GATA-3, which regulates the differentiation and expression of type-2 T-helper cell cytokines in allergic diseases [9].

The molecular pathways involved in regulation of inflammatory gene expression are now being delineated, and it is becoming clear that chromatin remodelling plays a critical role in the transcriptional control of genes. Stimuli that switch on inflammatory genes do so by changing the chromatin structure of the gene, whereas corticosteroids reverse this process.

CHROMATIN REMODELLING AND GENE EXPRESSION

Chromatin consists of DNA and basic proteins called histones, which provide the structural backbone of the chromosome. It has long been recognised that histones play a critical role in regulating the expression of genes and determine which genes are transcriptionally active and which ones are suppressed (silenced) [10]. The chromatin structure is highly organised since almost 2 m of DNA have to be packed into each cell nucleus. Chromatin is made up of nucleosomes, which are particles consisting of 146 base pairs of DNA wound almost twice around an octamer of two molecules each of the core histone proteins H2A, H2B, H3 and H4. Since the mid-1990s, it has been possible to elucidate how expression and repression of genes are associated with remodelling of this chromatin structure by enzymatic modification of the core histone proteins, particularly by acetylation. Each core histone has a long N-terminal tail that is rich in lysine residues, which may become acetylated, thus changing the electrical charge of the core histone. In the resting cell, DNA is wound tightly around core histones, excluding the binding of the enzyme RNA polymerase II, which activates gene transcription and the formation of mRNA. This conformation of the chromatin structure is described as closed and is associated with suppression of gene expression. Gene transcription occurs only when the chromatin structure is opened up, with unwinding of DNA so that RNA polymerase II and basal transcription complexes can bind to DNA to initiate transcription.

Histone acetyltransferases and coactivators

When pro-inflammatory transcription factors, such as NF-κB, are activated, they bind to specific recognition sequences in DNA and subsequently interact with large coactivator molecules, such as cAMP-response-element-binding protein (CREB)-binding protein (CBP), p300 and p300/CBP-associated factor (pCAF). These coactivator molecules act as the molecular switches that control gene transcription and all have intrinsic histone acetyltransferase (HAT) activity [11, 12]. This results in acetylation of core histones, thereby reducing their charge, which allows the chromatin structure to transform from the resting closed conformation to an activated open form [12]. This results in unwinding of DNA, and binding of TATA-box-binding protein and associated factors and RNA polymerase II, which then initiate gene transcription. This molecular mechanism is common to all genes, including those involved in differentiation, proliferation and activation of cells. Of course this process is reversible, and deacetylation of acetylated histones is associated with gene silencing. This is mediated by histone deacetylases (HDACs), which act as corepressors, together with other corepressor proteins that are subsequently recruited.

These fundamental mechanisms have now been applied to understanding the regulation of inflammatory genes in diseases such as asthma and COPD [13]. In a human epithelial cell line, activation of NF-κB, by exposing the cell to inflammatory signals such as interleukin (IL)-1β, tumour necrosis factor (TNF)-α or endotoxin, results in acetylation of specific lysine residues on histone H4 (the other histones do not appear to be so markedly or rapidly acetylated) and this correlates with increased expression of genes encoding inflammatory proteins, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) [14].

Histone deacetylases and corepressors

The acetylation of histone that is associated with increased expression of inflammatory genes is counteracted by the activity of HDACs, of which 11 that deacetylate histones are now characterised in mammalian cells [15, 16]. There is evidence that the different HDACs target different patterns of acetylation [17]. In biopsy specimens from patients with asthma, there is an increase in HAT and a reduction in HDAC activity, thereby favouring increased inflammatory gene expression [18]. Against this background, it is now possible to better understand why corticosteroids are so effective in suppressing this complex inflammatory process that involves the increased expression of multiple inflammatory proteins. HDACs act as corepressors in consort with other corepressor proteins, such as nuclear receptor corepressor and silencing mediator of retinoid and thyroid receptors, forming a corepressor complex that silences gene expression [19].

GLUCOCORTICOID RECEPTORS

Corticosteroids diffuse readily across cell membranes and bind to glucocorticoid receptors (GRs) in the cytoplasm. Cytoplasmic GRs are normally bound to proteins, known as molecular chaperones, such as heat shock protein 90 and FK-binding protein, which protect the receptor and prevent its nuclear localisation by covering the sites on the receptor that are needed for transport across the nuclear membrane into the

nucleus [20]. A single gene encodes human GR, but several variants are now recognised, as a result of alternative transcript splicing and alternative translation initiation [21]. GRα binds corticosteroids, whereas GRβ is an alternatively spliced form that binds to DNA but cannot be activated by corticosteroids. GRβ exhibits a very low level of expression compared to GRα [22]. The GRβ isoform has been implicated in steroid resistance in asthma [23], although whether GRβ can have any functional significance has been questioned in view of the very low levels of expression compared to those of GRα [24].

GRs may also be modified by phosphorylation and other modifications, which might alter the response to corticosteroids by affecting ligand binding, translocation to the nucleus, *trans*-activating efficacy, protein–protein interactions or recruitment of cofactors [25, 26]. For example, there are a number of serine/threonine residues in the N-terminal domain at which GR may be phosphorylated by various kinases.

Once corticosteroids have bound to GRs, changes in receptor structure result in dissociation of molecular chaperone proteins, thereby exposing nuclear localisation signals on the GR. This results in rapid transport of the activated GR–corticosteroid complex into the nucleus, where it binds to DNA at specific sequences in the promoter region of corticosteroid-responsive genes known as glucocorticoid response elements (GREs). Two GR molecules come together as a homodimer and bind to GRE, leading to changes in gene transcription. Interaction of GRs with GREs classically leads to an increase in gene transcription (*trans*-activation), but negative GRE sites have also been described, at which binding of GR leads to gene suppression (*cis*-repression) (fig. 1) [27]. There are few well-documented examples of negative GREs, but some are relevant to corticosteroid side-effects, including genes that regulate the hypothalamopituitary axis (proopiomelanocortin and corticotrophin-releasing factor), bone metabolism (osteocalcin) and skin structure (keratins).

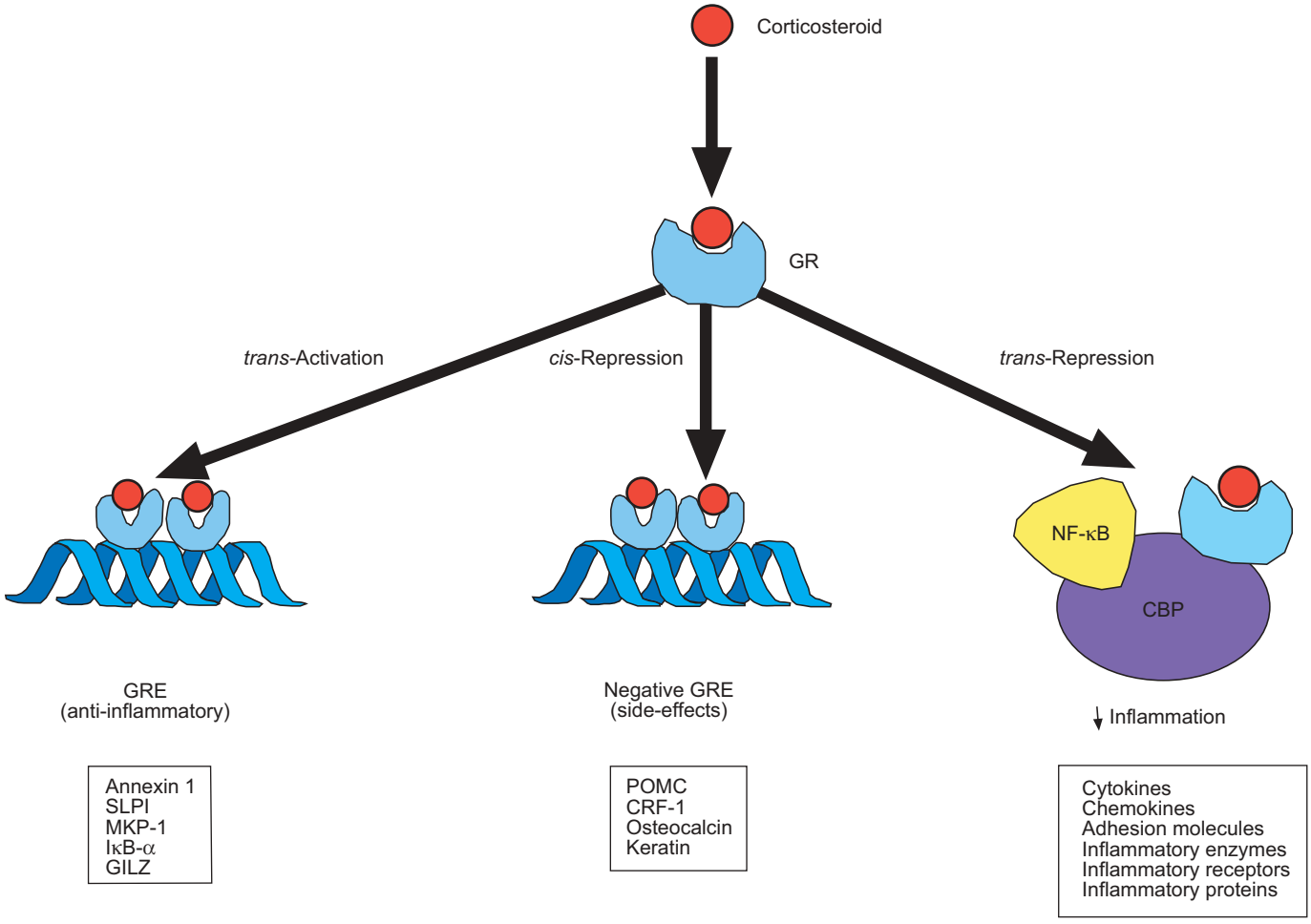

FIGURE 1. Corticosteroids may regulate gene expression in several ways. Corticosteroids enter the cell to bind to glucocorticoid receptors (GRs) in the cytoplasm that translocate to the nucleus. GR homodimers bind to glucocorticoid response elements (GREs) in the promoter region of steroid-sensitive genes, which may encode anti-inflammatory proteins. Less commonly, GR homodimers interact with negative GREs to suppress genes, particularly those linked to side-effects of corticosteroids. Nuclear GRs also interact with coactivator molecules, such as cAMP-response-element-binding-protein-binding protein (CBP), which is activated by pro-inflammatory transcription factors, such as nuclear factor (NF)-κB, thus switching off the inflammatory genes that are activated by these transcription factors. SLPI: secretory leukoprotease inhibitor; MKP: mitogen-activated protein kinase phosphatase; IκB-α: inhibitor of NF-κB; GILZ: glucocorticoid-induced leucine zipper protein; POMC: pro-opiomelanocortin; CRF: corticotrophin releasing factor. ↓: decrease.

CORTICOSTEROID-INDUCED GENE TRANSCRIPTION

Corticosteroids produce their effect on responsive cells by activating GRs in order to directly or indirectly regulate the transcription of target genes. The number of genes per cell directly regulated by corticosteroids is estimated to be 10–100, but many genes are indirectly regulated through interaction with other transcription factors and coactivators. GR homodimers bind to GRE sites in the promoter region of corticosteroid-responsive genes. Interaction of the activated GR dimer with a GRE usually increases transcription. GRs may increase transcription by interacting with coactivator molecules, such as CBP and pCAF, thus activating histone acetylation and gene transcription. For example, relatively high concentrations of corticosteroids increase secretion of the antiprotease secretory leukoprotease inhibitor (SLPI) from epithelial cells [14].

The activation of genes by corticosteroids is associated with selective acetylation of lysine residues 5 and 16 on histone H4, resulting in increased gene transcription (fig. 2) [14, 28]. Activated GRs may bind to coactivator molecules, such as CBP and pCAF, as well as steroid receptor coactivator (SRC)-1 and glucocorticoid receptor-interacting protein 1 (GRIP1 or SRC-2), which all possess HAT activity [29, 30]. GRs preferentially associate with GRIP1/SRC-2, which subsequently recruits pCAF [31].

Anti-inflammatory gene activation

Several of the genes that are switched on by corticosteroids have anti-inflammatory effects, including annexin 1 (lipocortin-1), SLPI, IL-10 and the inhibitor of NF-κB (IκB-α). However, therapeutic doses of inhaled corticosteroids have not been shown to increase annexin 1 concentrations in

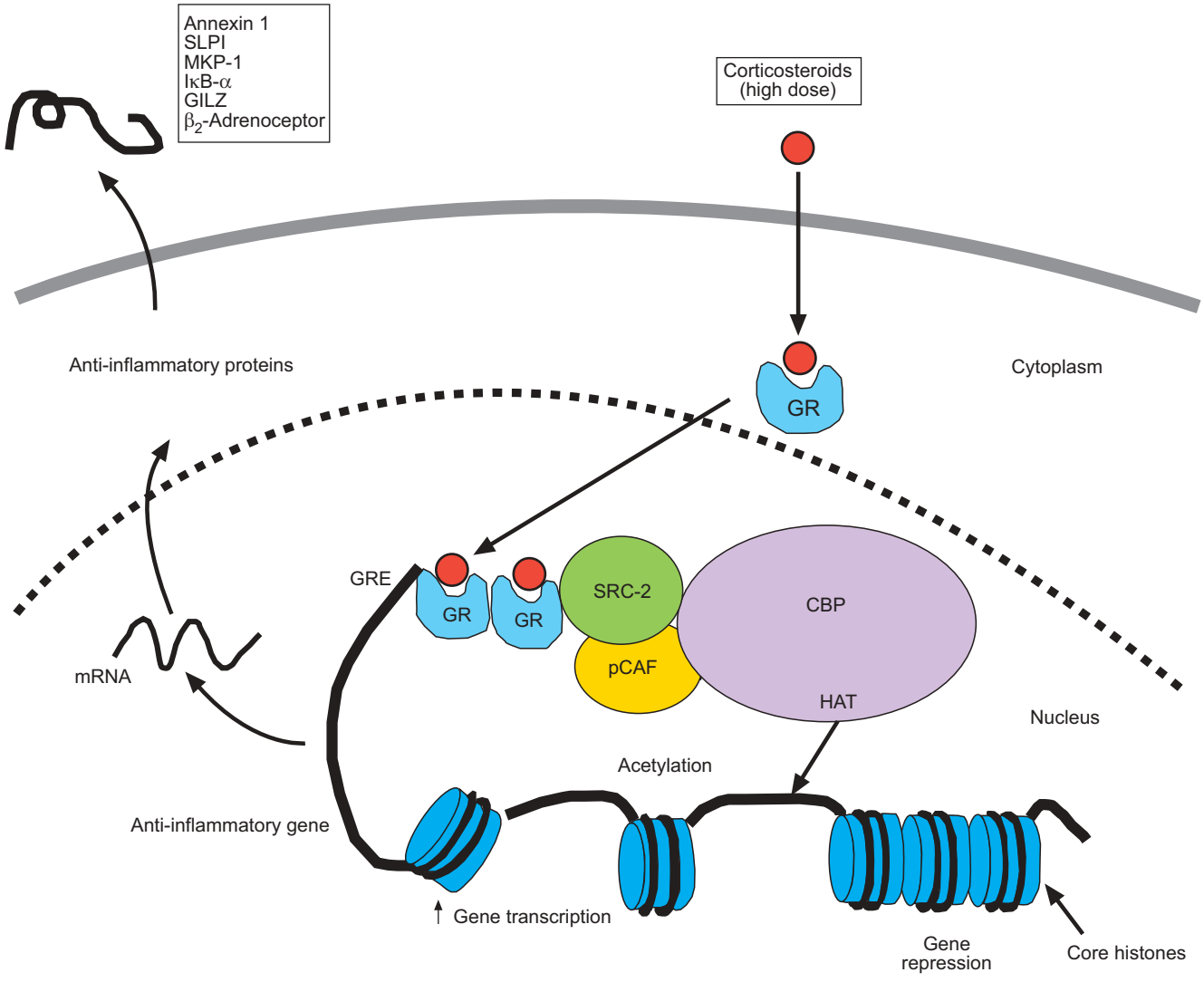

FIGURE 2. Corticosteroid activation of anti-inflammatory gene expression. Corticosteroids bind to cytoplasmic glucocorticoid receptors (GRs) that translocate to the nucleus, where they bind to glucocorticoid response elements (GREs) in the promoter region of steroid-sensitive genes and also directly or indirectly to coactivator molecules such as cAMP-response-element-binding-protein-binding protein (CBP), p300/CBP-associated factor (pCAF) or steroid receptor coactivator (SRC)-2, which have intrinsic histone acetyltransferase (HAT) activity, causing acetylation of lysines on histone H4, which leads to activation of genes encoding anti-inflammatory proteins, such as secretory leukoprotease inhibitor (SLPI), mitogen-activated protein kinase phosphatase (MKP)-1, inhibitor of nuclear factor-κB (IκB-α) and glucocorticoid-induced leucine zipper protein (GILZ). ↑: increase.

bronchoalveolar lavage fluid [32], and an increase in IκB-α level has not been shown in most cell types, including epithelial cells [33, 34]. Corticosteroids also switch on the synthesis of two proteins that affect inflammatory signal transduction pathways, glucocorticoid-induced leucine zipper protein, which inhibits both NF-κB and AP-1 [35], and mitogen-activated protein (MAP) kinase phosphatase (MKP)-1, which inhibits p38 MAP kinase [36]. However, it seems unlikely that the widespread anti-inflammatory actions of corticosteroids could be entirely explained by increased transcription of small numbers of anti-inflammatory genes, particularly since high concentrations of corticosteroids are usually required for this effect, whereas, in clinical practice, corticosteroids are able to suppress inflammation at low concentrations.

Side-effect gene repression

Relatively little is known about the molecular mechanisms of corticosteroid side-effects, such as osteoporosis, growth retardation in children, skin fragility and metabolic effects. These actions of corticosteroids are related to their endocrine effects. The systemic side-effects of corticosteroids may be due to gene activation. Some insight into this has been provided by mutant GRs that do not dimerise and, therefore, cannot bind to GREs to switch on genes. In transgenic mice expressing these mutant GRs, corticosteroids show no loss of anti-inflammatory effect and are able to suppress NF-κB-activated genes in the normal way [37]. As indicated above, several of the genes associated with side-effects, including the hypothalamopituitary axis, bone metabolism and skin structure, appear to be regulated by interaction of GRs with negative GRE sites [26].

SWITCHING OFF INFLAMMATORY GENES

In controlling inflammation, the major effect of corticosteroids is to inhibit the synthesis of multiple inflammatory proteins through suppression of the genes that encode them (table 1). Although this was originally believed to be through interaction of GRs with negative GRE sites, these have been demonstrated on only a few genes, which do not include those encoding inflammatory proteins [26].

Interaction with transcription factors

Activated GRs have been shown to interact functionally with other activated transcription factors. Most of the inflammatory genes that are activated in asthma do not have GRE sites in their promoter regions, yet are potently repressed by corticosteroids. There is persuasive evidence that corticosteroids inhibit the effects of pro-inflammatory transcription factors, such as AP-1 and NF-κB, which regulate the expression of genes that code for many inflammatory proteins, such as cytokines, inflammatory enzymes, adhesion molecules and inflammatory receptors [38, 39]. Activated GRs can interact directly with other activated transcription factors by protein–protein binding, but this may be a particular feature of cells in which these genes are artificially overexpressed, rather than a property of normal cells. Treatment of asthmatic patients with high doses of inhaled corticosteroids that suppress airway inflammation is not associated with any reduction in NF-κB binding to DNA, yet is able to switch off inflammatory genes, such as GM-CSF, that are regulated by NF-κB [40]. This suggests that corticosteroids are more likely to be acting downstream of the binding of pro-inflammatory transcription factors to DNA, and attention has now focused on their effects on chromatin structure and histone acetylation.

TABLE 1 Effect of corticosteroids on gene transcription

Increased transcription (*trans*-activation)
Annexin 1 (lipocortin-1, phospholipase A_2 inhibitor)
β_2-Adrenergic receptor
Secretory leukoprotease inhibitor
CC10 (phospholipase A_2 inhibitor)
IL-1 receptor antagonist
IL-1 receptor type II (decoy receptor)
IκB-α
GILZ
MKP-1
IL-10 (indirectly)
Decreased transcription (*trans*-repression)
Cytokines
IL-1 to IL-6, IL-9, IL-11 to IL-13, IL-16 to IL-18, TNF-α, GM-CSF, SCF
Chemokines
IL-8, RANTES, MIP-1α, MCP-1, MCP-3, MCP-4, eotaxins
Adhesion molecules
ICAM-1, VCAM-1, E-selectin
Inflammatory enzymes
iNOS, inducible COX-2, $cPLA_2$
Inflammatory receptors
Tachykinin NK_1 and NK_2 receptors, bradykinin B_2 receptors
Peptides
Endothelin-1

CC10: clara cell 10-kDa protein; IL: interleukin; IκB-α: inhibitor of nuclear factor-κB; GILZ: glucocorticoid-induced leucine zipper protein; MKP: mitogen-activated protein kinase phosphatase; TNF: tumour necrosis factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; SCF: stem cell factor; RANTES: regulated on activation, normal T-cell expressed and secreted; MIP: macrophage inflammatory protein; MCP: monocyte chemoattractant protein; ICAM: intercellular adhesion molecule; VCAM: vascular cell adhesion molecule; iNOS: inducible nitric oxide synthase; $cPLA_2$: cytoplasmic phospholipase A_2.

Effects on histone acetylation

Repression of genes occurs through reversal of the histone acetylation that switches on inflammatory genes [41]. Activated GRs may bind to CBP or other coactivators directly to inhibit their HAT activity [14], thus reversing the unwinding of DNA around core histones, and thereby repressing inflammatory genes. More importantly, particularly at low concentrations that are likely to be relevant therapeutically in asthma treatment, activated GR recruits HDAC2 to the activated transcriptional complex, resulting in deacetylation of histones, and, thus, a decrease in inflammatory gene transcription (fig. 3) [14]. Using a chromatin immunoprecipitation assay, it has been demonstrated that corticosteroids recruit HDAC2 to the acetylated histone H4 associated with the GM-CSF promoter [14]. Using RNA interference to selectively suppress HDAC2 in an epithelial cell line, it has been shown that there is an increase in the expression of GM-CSF and reduced sensitivity to corticosteroids [42]. By contrast,

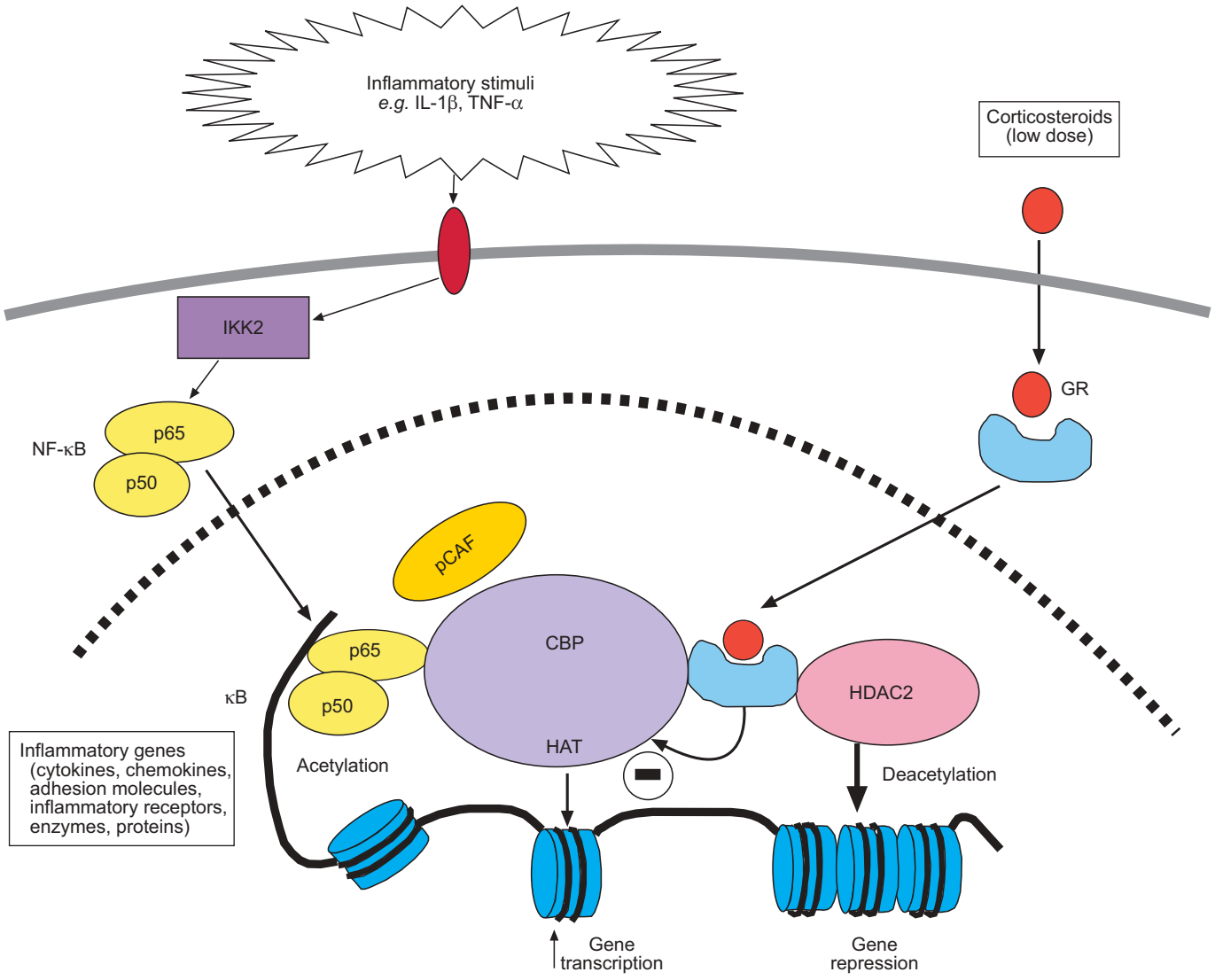

FIGURE 3. Corticosteroid suppression of activated inflammatory genes. Inflammatory genes are activated by inflammatory stimuli, such as interleukin (IL)-1β or tumour necrosis factor (TNF)-α, resulting in activation of inhibitor of I-κB kinase (IKK)2, which activates the transcription factor nuclear factor (NF)-κB. A dimer of p50 and p65 NF-κB translocates to the nucleus and binds to specific κB recognition sites and also to coactivators, such as cAMP-response-element-binding-protein-binding protein (CBP) or p300/CBP-associated factor (pCAF), which have intrinsic histone acetyltransferase (HAT) activity. This results in acetylation of core histone H4, resulting in increased expression of genes encoding multiple inflammatory proteins. Glucocorticoid receptors (GRs), after activation by corticosteroids, translocate to the nucleus and bind to coactivators in order to inhibit HAT activity directly and recruiting histone deacetylase (HDAC)2, which reverses histone acetylation, leading to suppression of these activated inflammatory genes. ↑: increase; -: suppression.

knock-down of HDAC1 and HDAC3 had no such effect on steroid responsiveness. An important issue that is not yet resolved is why corticosteroids selectively switch off inflammatory genes, while having no effect on genes that regulate proliferation, metabolism and survival. It is likely that GRs only bind to coactivators that are activated by pro-inflammatory transcription factors, such as NF-κB and AP-1, although it is not yet understood how this specific recognition occurs. AP-1 and NF-κB repression is normal in mice which express a form of GR that does not dimerise ($dim^{-/-}$), indicating that GR monomers are able to mediate the anti-inflammatory effects of corticosteroids, whereas dimerisation is needed for gene activation responses [37, 43].

Other histone modifications

It has now become apparent that core histones may be modified not only by acetylation but also by methylation, phosphorylation and ubiquitination, and that these modifications may also regulate gene transcription [44, 45]. Methylation of histones, particularly histone H3, by histone methyltransferases, usually results in gene suppression [46]. The anti-inflammatory effects of corticosteroids are reduced by a methyltransferase inhibitor, 5-aza-2′-deoxycytidine, suggesting that this may be an additional mechanism whereby corticosteroids suppress genes [47]. Indeed, there may be an interaction between acetylation, methylation and phosphorylation of histones, such that the sequence of chromatin

modifications (the so-called histone code) may give specificity to expression of particular genes [48–50].

Nontranscriptional effects

Although most of the actions of corticosteroids are mediated by changes in transcription through chromatin remodelling, it is increasingly recognised that they may also affect protein synthesis by reducing the stability of mRNA such that less protein is synthesised. It is increasingly recognised that several inflammatory proteins are regulated post-transcriptionally at the level of mRNA stability [51]. This may be an important anti-inflammatory mechanism as it allows corticosteroids to switch off the ongoing production of inflammatory proteins after the inflammatory gene has been activated. The stability of some inflammatory genes is determined by regulation of adenine–uracil (AU)-rich elements (ARE) in the 3′-untranslated regions of the gene which interact with several ARE-binding proteins, such as Hu antigen R (HuR) and tristetraprolin, that may stabilise mRNA [52, 53]. Some inflammatory genes, such as the genes encoding GM-CSF and cyclooxygenase (COX)-2, produce mRNA that is particularly susceptible to the action of ribonucleases, which break down mRNA, thus switching off protein synthesis. Corticosteroids may have inhibitory effects on the proteins that stabilise mRNA, leading to more rapid breakdown and, thus, a reduction in inflammatory protein expression [54–56]. Corticosteroids do not appear to have any effect on HuR or tristeraprolin expression, however [57].

EFFECTS ON SIGNAL TRANSDUCTION PATHWAYS

Corticosteroids have complex effects on signal transduction pathways through *trans*-repression of critical enzymes involved in inflammatory cascades, or through increased transcription of endogenous inhibitors of these pathways.

Mitogen-activated protein kinase pathways

MAP kinases play an important role in inflammatory gene expression through the regulation of pro-inflammatory transcription factors [58]. There is increasing evidence that corticosteroids may exert an inhibitory effect on these pathways. Corticosteroids may inhibit AP-1 and NF-κB *via* an inhibitory effect on c-Jun N-terminal kinases (JNKs), which activate these transcription factors [59, 60]. Corticosteroids reduce the stability of mRNA for some inflammatory genes, such as COX-2, through an inhibitory action on another MAP kinase, p38 MAP kinase [53, 61]. p38 MAP kinase regulates multiple inflammatory genes, including TNF-α, IL-1β, IL-6, GM-CSF and IL-8, which have ARE sites in their 3′-untranslated regions, by stabilising their mRNA such that synthesis of the inflammatory protein is increased [53]. The inhibitory effect of corticosteroids is mediated *via* the rapid induction of a potent endogenous inhibitor of p38 MAP kinase, MKP-1, which is one of the genes switched on by corticosteroids (fig. 4) [36, 62]. In gene microarray studies, MKP-1 is one of the most prominent genes activated by corticosteroids [63]. Corticosteroids not only induce the MKP-1 gene but also reduce its degradation [64]. MKP-1 inhibits all MAP kinase pathways and therefore inhibits JNK, and, to a lesser extent, extracellular signal-regulated kinase, in addition to p38 MAP kinase [62]. This indicates that corticosteroids have the capacity to inhibit all MAP kinase pathways, but the selectivity of MKP-1 for different MAP kinases appears to vary from cell to cell [65]. The effect of a corticosteroid on MKP-1 expression is enhanced by low concentrations of both salmeterol and formoterol [66]. This may contribute to the enhanced anti-inflammatory effects of corticosteroids induced by long-acting β_2-agonists in combination therapy. At least 10 other MAP kinase phosphatases have now been identified, with differing cellular distributions and selectivity, but it is not yet certain whether or not they are induced by corticosteroids [67].

CORTICOSTEROID RESISTANCE

Although corticosteroids are highly effective in the control of asthma and other chronic inflammatory or immune diseases, a small proportion of patients with asthma fail to respond to even high doses of oral corticosteroids [68–70], and patients with COPD are largely unresponsive to corticosteroids [71]. Resistance to the therapeutic effects of corticosteroids is also recognised in nonpulmonary inflammatory and immune diseases, including rheumatoid arthritis and inflammatory bowel disease. Corticosteroid-resistant patients present considerable management problems as there are few alternative anti-inflammatory treatments available. The new insights into the mechanisms whereby corticosteroids suppress chronic inflammation have shed light on the molecular basis of corticosteroid resistance in asthma and COPD.

Steroid-resistant asthma

There may be several molecular mechanisms of resistance to the effects of corticosteroids and these may differ between patients [1, 69, 70]. It is likely that there is a spectrum of steroid responsiveness, with the very rare resistance at one end, and with relative resistance seen in patients who require high doses of inhaled and oral steroids (steroid-dependent asthma).

p38 mitogen-activated protein kinase

Biopsy studies have demonstrated the typical eosinophilic inflammation found in the bronchial mucosa of such patients, with increased expression of type-2 T-helper cell cytokines [72]. There is also resistance to the anti-inflammatory effects of corticosteroids in circulating mononuclear cells [73–75]. Certain cytokines (particularly IL-2, IL-4 and IL-13, which show increased expression in bronchial biopsy specimens from patients with steroid-resistant asthma) may induce a reduction in the affinity of GRs in inflammatory cells such as T-lymphocytes, resulting in local resistance to the anti-inflammatory actions of corticosteroids [68, 76]. The combination of IL-2 and IL-4 induces steroid resistance *in vitro* through activation of p38 MAP kinase, which phosphorylates GRs and reduces corticosteroid binding affinity within the nucleus [77]. The therapeutic implication is that p38 MAP kinase inhibitors now in clinical development might reverse this form of steroid resistance.

Glucocorticoid receptor β

Another proposed mechanism for steroid resistance in asthma is increased expression of GRβ, which may theoretically act as an inhibitor by competing with GRα for binding to GRE sites or interacting with coactivator molecules [78]. However, there is no increased expression of GRβ in the mononuclear cells of patients with steroid-dependent asthma, which show reduced responsiveness to corticosteroids *in vitro*. Furthermore, GRα

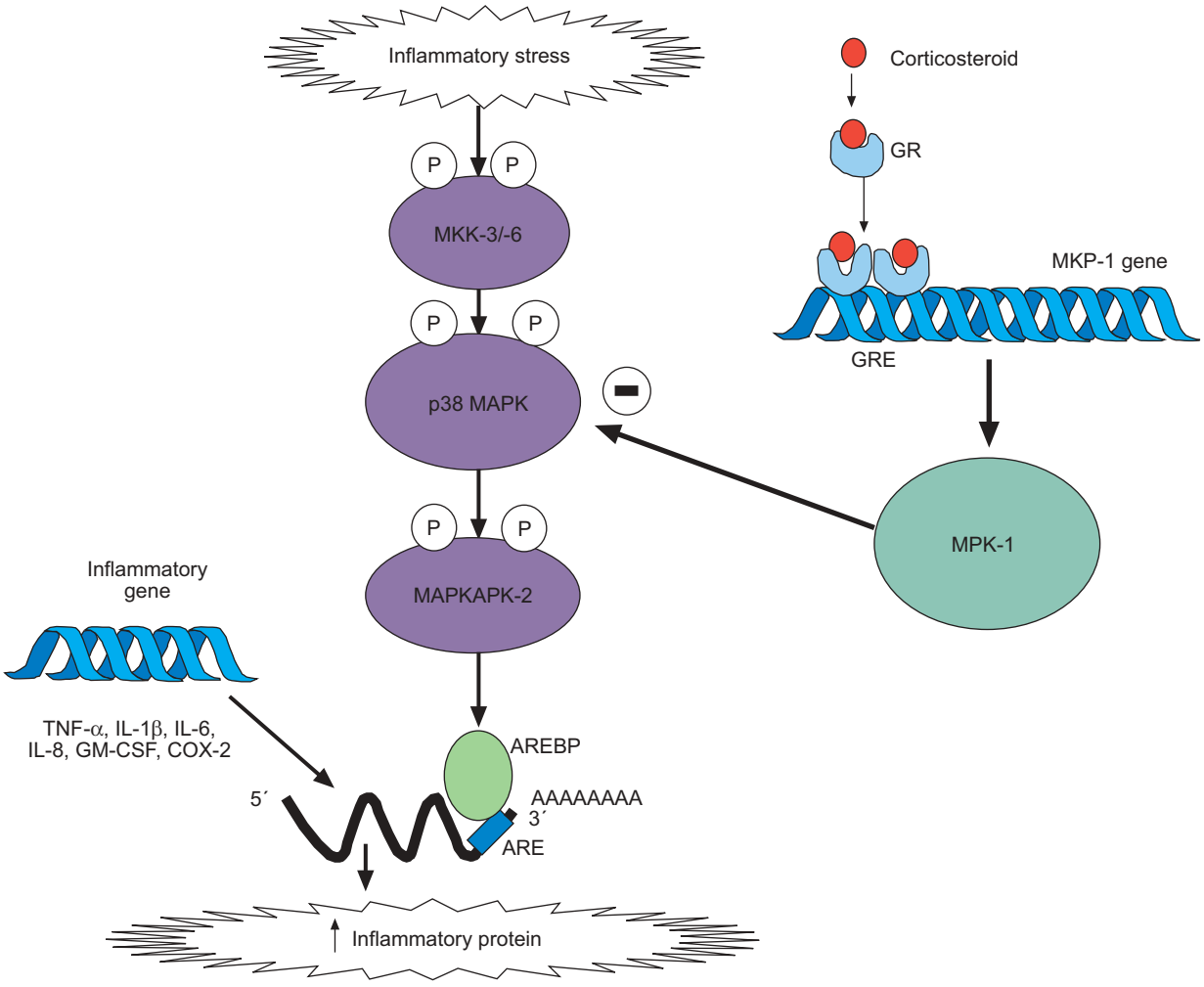

FIGURE 4. Inhibition of p38 mitogen-activated protein kinase (MAPK) by corticosteroids. p38 MAPK is activated by inflammatory stresses though activation of MAPK kinase (MKK)-3 and -6. p38 phosphorylates (P) MAPK-activated protein kinase (MAPKAPK)-2, which plays a role in stabilising mRNA encoding several inflammatory proteins, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and cyclooxygenase (COX)-2. This mRNA is characterised by adenine–uracil-rich elements (AREs) in the 3′-untranslated region, which make the mRNA unstable and rapidly degraded. ARE-binding proteins (AREBPs) stabilise these proteins and may be activated (probably indirectly) by MAPKAPK-2. Corticosteroids induce the expression of MAPK phosphatase (MKP)-1, which inhibits p38 and, thus, prevents the stabilisation of multiple inflammatory proteins. GR: glucocorticoid receptor; GRE: glucocorticoid response element. ↑: increase; -: suppression.

greatly predominates over GRβ, making it unlikely that it could have any functional inhibitory effect [79, 80], and GRβ is undetectable in the blood monocytes of asthmatic patients [81]. Furthermore, there is no evidence for induction of GRβ in response to IL-2/IL-4 exposure, which induces corticosteroid resistance in mononuclear cells, convincingly demonstrating that GRβ cannot account for corticosteroid resistance in asthma [81].

Interaction with transcription factors

Another proposed mechanism is a failure of GRs to inhibit the activation of inflammatory genes by transcription factors such as NF-κB and AP-1. Indeed, there is defective inhibition of AP-1 in response to corticosteroid in the mononuclear cells of steroid-resistant patients [75]. This may be caused by increased activation of AP-1 due to excessive activation of the JNK pathway, which has been demonstrated in the cells of steroid-resistant asthma patients [82].

Defective histone acetylation

Mononuclear cells from asthmatic patients who are steroid-dependent or -resistant show reduced suppression of cytokine release and a reduction in histone H4 acetylation in the nucleus following treatment with a high concentration (1 μM) of dexamethasone [83]. In one group of patients, nuclear localisation of GRs in response to a high concentration of corticosteroids was impaired, and this accounts for the reduced histone acetylation, since there is a direct correlation between the degree of histone acetylation and GR nuclear localisation [83]. This may be a result of GR nitrosylation, leading to reduced dissociation of GR from heat shock protein 90 [84]. However, in another group of patients, the defect in

histone acetylation is found despite normal nuclear localisation of GRs. This may be a result of GR phosphorylation within the nucleus due to the activation of p38 MAP kinase [77], which may result in a failure to recruit a distinct coactivator(s). This may result in failure of GRs to *trans*-activate steroid-responsive genes [85]. In this group of patients, specific acetylation of histone H4 lysine 5 by corticosteroids is defective [83]. This presumably means that corticosteroids are not able to activate certain genes that are critical to the anti-inflammatory action of high doses of corticosteroids, but whether or not this is a rare genetic defect is not yet known.

Smoking asthmatics

Asthmatic patients who smoke have more severe disease and are also resistant to the anti-inflammatory effects of corticosteroids [86, 87]. A plausible explanation for this steroid resistance is the combined effect of asthma and cigarette smoking on HDAC, resulting in a marked reduction comparable to that seen in COPD patients, and this is confirmed by preliminary data [88].

Corticosteroid resistance in COPD

Although inhaled corticosteroids are highly effective in asthma, they provide relatively little therapeutic benefit in COPD, despite the fact that active airway and lung inflammation is present. This may reflect the fact that the inflammation in COPD is not suppressed by corticosteroids, with no reduction in inflammatory cell numbers or cytokine or protease levels in induced sputum even with oral corticosteroids [89–91]. Furthermore, histological analysis of peripheral airways of patients with severe COPD shows an intense inflammatory response, despite treatment with high doses of inhaled corticosteroids [92]. There is increasing evidence for an active steroid resistance mechanism in COPD, since corticosteroids fail to inhibit cytokines (such as IL-8 and TNF-α) that they normally suppress [89, 90]. *In vitro* studies show that cytokine release from alveolar macrophages is markedly resistant to the anti-inflammatory effects of corticosteroids, compared to that from cells from normal smokers, and these, in turn, are more resistant than that from alveolar macrophages from nonsmokers [93, 94]. This lack of response to corticosteroids may be explained, at least in part, by an inhibitory effect of cigarette smoking and oxidative stress on HDAC function, thus interfering with the critical anti-inflammatory action of corticosteroids [95]. Indeed, there is a correlation between HDAC activity and the suppressive effects of a corticosteroid on cytokine release. It is likely that oxidative and nitrative stress in COPD specifically impair HDAC2 [96], resulting in corticosteroid resistance (fig. 5) [3]. Although this is seen in all stages of COPD, it is most marked in the patients with the most severe disease [97]. Even in patients with COPD who have stopped smoking, the steroid resistance persists [89, 90], and these patients are known to experience continuing oxidative stress [98].

Oxidative stress is also increased in patients with severe asthma and during exacerbations [99–101], such that a reduction in HDAC may also account for the reduced responsiveness to corticosteroids in these patients and the relative unresponsiveness of acute exacerbation of asthma to corticosteroids.

THERAPEUTIC IMPLICATIONS

Inhaled corticosteroids are now used as first-line therapy for the treatment of persistent asthma in adults and children in many countries, since they are the most effective treatment for asthma currently available [102]. However, at high doses, systemic absorption of inhaled corticosteroids may have deleterious effects, and so there has been a search for safer steroids for inhalation and even for oral administration.

Dissociated corticosteroids

The currently available inhaled corticosteroids are absorbed from the lungs into the systemic circulation, and, therefore, inevitably have some systemic component. Understanding the molecular mechanisms of action of corticosteroids has led to the development of a new generation of corticosteroids. The major task in developing these drugs is to dissociate the anti-inflammatory effects from the endocrine actions that are associated with side-effects. As discussed above, a major mechanism of the anti-inflammatory effect of corticosteroids appears to be inhibition of the effects of pro-inflammatory transcription factors, such as NF-κB and AP-1, which are activated by pro-inflammatory cytokines (*trans*-repression) *via* an inhibitory action on histone acetylation and stimulation of histone deacetylation, without DNA binding. In contrast, the endocrine and metabolic effects of steroids that are responsible for the systemic side-effects of corticosteroids are likely to be mediated predominantly *via* DNA binding through interaction of GRs with negative GREs (*cis*-repression). This has led to a search for novel corticosteroids that selectively *trans*-repress without significant *trans*-activation or *cis*-repression, thus reducing the potential risk of systemic side-effects. Since corticosteroids bind to the same GR, this seems at first to be an unlikely possibility, but, although DNA binding involves a GR homodimer, interaction with transcription factors AP-1 and NF-κB and coactivators involves only a single GR [28]. A separation of *trans*-activation and *trans*-repression has been demonstrated using reporter gene constructs with selective mutations of the GR in transfected cells [103]. In addition, in mice with GRs that do not dimerise, there is no *trans*-activation, but *trans*-repression appears to be normal [37, 43]. Furthermore, some steroids, such as the antagonist RU486, exhibit greater *trans*-repression than *trans*-activation effects. Indeed, the topical steroids used in asthma therapy today, such as fluticasone propionate and budesonide, appear to show more potent *trans*-repression than *trans*-activation effects, which may account for their selection as potent anti-inflammatory agents [104, 105]. Recently, a novel class of steroids has been described, in which there is potent *trans*-repression with relatively little *trans*-activation. These dissociated steroids, including RU24858 and RU40066, have anti-inflammatory effects *in vitro* [106], although there is little separation of anti-inflammatory effects and systemic side-effects *in vivo* [107]. Other dissociated corticosteroids appear to show dissociation *in vivo* [108]. Several dissociated corticosteroids are now in clinical development and may lead to inhaled steroids with greater safety, or even to oral steroids which are less likely to produce significant adverse effects. The recent resolution of the crystal structure of the ligand-binding domain of GRs may help in the better design of dissociated steroids [109].

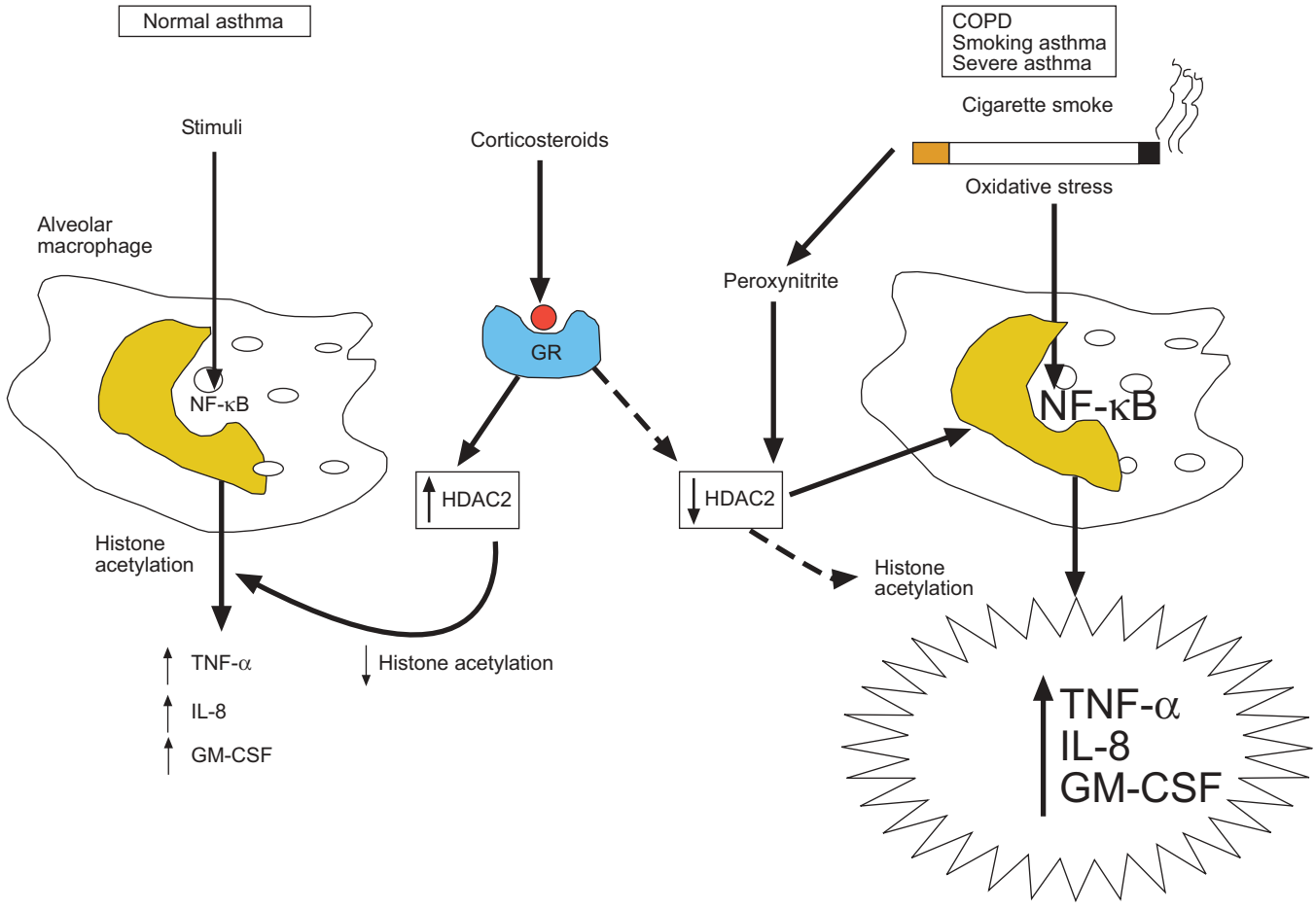

FIGURE 5. A proposed mechanism of corticosteroid resistance in chronic obstructive pulmonary disease (COPD), severe asthma and smoking asthma. Stimulation of normal and asthmatic alveolar macrophages activates nuclear factor (NF)-κB and other transcription factors to switch on histone acetyltransferase, leading to histone acetylation and, subsequently, transcription of genes encoding inflammatory proteins, such as tumour necrosis factor (TNF)-α, interleukin (IL)-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Corticosteroids reverse this by binding to glucocorticoid receptors (GRs) and recruiting histone deacetylase (HDAC)2. This reverses the histone acetylation induced by NF-κB and switches off the activated inflammatory genes. In COPD and smoking asthmatic patients, cigarette smoke generates oxidative stress (acting through the formation of peroxynitrite) to impair the activity of HDAC2. This amplifies the inflammatory response to NF-κB activation, but also reduces the anti-inflammatory effect of corticosteroids, since HDAC2 is now unable to reverse histone acetylation. A similar mechanism may operate in severe asthma, in which increased oxidative stress is generated by airway inflammation. ↑: increase; ↓: decrease.

Nonsteroidal anti-inflammatory treatments

Now that the molecular mechanisms of corticosteroid action have been elucidated, this raises the possibility that novel nonsteroidal anti-inflammatory treatments might be developed which mimic the actions of corticosteroids on inflammatory gene regulation. Many of the anti-inflammatory effects of corticosteroids appear to be mediated *via* inhibition of the transcriptional effects of NF-κB, and low-molecular-mass inhibitors of IKK2 (inhibitor of I-κB kinase-2) that activate NF-κB are now in preclinical development [110]. However, corticosteroids have effects in addition to inhibiting NF-κB-regulated genes, and so it is not certain whether or not IKK2 inhibitors will parallel the clinical effectiveness of corticosteroids, and they may have side-effects, such as increased susceptibility to infections.

As discussed above, some of the therapeutic effects of corticosteroid are mediated *via* inhibition of p38 MAP kinase, and this kinase is also involved in some cases of steroid resistance in asthma. Several p38 MAP kinase inhibitors are now in clinical development and these drugs might mimic some of the effects of corticosteroids [111, 112], but may be of particular value in asthmatic patients with corticosteroid resistance.

Reversal of corticosteroid resistance

In COPD and severe asthma and in asthmatic patients who smoke, the poor responsiveness to corticosteroids may reflect a reduction in HDAC2 activity, as discussed above. Theophylline represents the first drug that has been shown to activate HDAC, resulting in marked potentiation of the anti-inflammatory effects of corticosteroids [113, 114]. This action of theophylline is not mediated *via* phosphodiesterase inhibition or adenosine receptor antagonism, and, therefore, appears to be a novel action of the drug [113]. In COPD macrophages, low concentrations of theophylline are able to restore HDAC activity to normal and reverse the steroid resistance of these cells *in vitro* [114]. Clinical studies to explore this effect of

theophylline are now underway. It may be possible to discover other drugs in this class that could form the basis of a new class of anti-inflammatory drugs without the side-effects that limit the use of theophylline [115].

Since oxidative stress and peroxynitrite appear to inhibit HDAC activity and mimic the defect in HDAC seen in COPD patients, antioxidants or inhibitors of inducible nitric oxide synthase might be expected to be effective. New and more effective antioxidants are in development [116], and selective inducible nitric oxide synthase inhibitors are already in clinical trials [117].

CONCLUSIONS

Corticosteroids exert their anti-inflammatory effects through influencing multiple signal transduction pathways. Their most important action is switching off multiple activated inflammatory genes through inhibition of histone acetyltransferase and recruitment of histone deacetylase 2 activity to the inflammatory gene transcriptional complex. In addition, corticosteroids may activate several anti-inflammatory genes and increase the degradation of mRNA encoding certain inflammatory proteins. This broad array of actions may account for the striking efficacy of corticosteroids in complex inflammatory diseases such as asthma and the difficulty in finding alternative anti-inflammatory drugs. There is now a better understanding of how responsiveness to corticosteroids is reduced in severe asthma, asthmatic patients who smoke and patients with chronic obstructive pulmonary disease. An important mechanism now emerging is a reduction in histone deacetylase 2 activity as a result of oxidative stress. These new insights into corticosteroid action may lead to new approaches to treating inflammatory lung diseases and in particular to increasing efficacy of steroids in situations in which they are less effective.

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