Update on glucocorticoid action and resistance

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Extensive development of inhaled and oral glucocorticoids has resulted in highly potent molecules that have been optimized to target activity to the lung and minimize systemic exposure. These have proved highly effective for most asthmatic subjects, but despite these developments, there are a number of subjects with asthma who fail to respond to even high doses of inhaled or even oral glucocorticoids. Advances in delineating the fundamental mechanisms of glucocorticoid pharmacology, especially the concepts of transactivation and transrepression and cofactor recruitment, have resulted in better understanding of the molecular mechanisms whereby glucocorticoids suppress inflammation. The existence of multiple mechanisms underlying glucocorticoid insensitivity raises the possibility that this might indeed reflect different diseases with a common phenotype, and studies examining the efficacy of potential new agents should be targeted toward subgroups of patients with severe corticosteroid-resistant asthma who clearly require effective new drugs and other approaches to improved asthma control. (J Allergy Clin Immunol 2006;117:522-43.)

Key words: *Severe asthma, steroid resistance, glucocorticoid receptor, molecular mechanisms, future therapies*

All patients with asthma have a specific pattern of inflammation in the airways that is characterized by degranulated mast cells, an infiltration of eosinophils, and an increased number of activated T_H2 cells.[1] It is believed that this specific pattern of inflammation underlies the clinical features of asthma, including intermittent wheezing, dyspnea, cough, and chest tightness. Approximately 100 known inflammatory mediators are increased in asthma and include lipid mediators, inflammatory peptides, chemokines, cytokines, and growth factors.[2] There is increasing evidence that structural cells of the airways, such as epithelial cells, airway smooth muscle cells, endothelial cells, and fibroblasts, are a major source of inflammatory mediators in asthma.[3]

Suppression of this inflammation by glucocorticoids controls and prevents these symptoms in the vast majority of patients,[4] and if used appropriately, these patients usually have no problems in terms of adverse effects. However, 5% to 10% of asthmatic patients do not respond

Abbreviations used

AHR: Airway hyperresponsiveness
AP-1: Activator protein 1
ARE: Adenylate-uridylate–rich element
BAL: Bronchoalveolar lavage
CBP: Cyclic AMP response element binding protein (CREB) binding protein
CD: Corticosteroid dependent
COPD: Chronic obstructive pulmonary disease
CR: Corticosteroid resistant
CS: Corticosteroid sensitive
GR: Glucocorticoid receptor
GRE: Glucocorticoid response element
HAT: Histone acetyltransferase
HDAC: Histone deacetylase
HFA: Hydrofluoroalkane
HuR: Hu antigen R
ERK: Extracellular signal–regulated kinase
IκBα: Inhibitor of nuclear factor κB
IKK: Inhibitor of NF-κB kinase
IRF-3: Interferon response factor 3
JAK: Janus-associated kinase
JNK: c-Jun N-terminal kinase
Kd: Dissociation constant
LBD: Ligand binding domain
MAPK: Mitogen-activated protein kinase
MKP-1: Mitogen-activated protein kinase phosphatase 1
NF-κB: Nuclear factor κB
NLS: Nuclear localization sequence
NO: Nitric oxide
Nrf2: Nuclear erythroid 2 p45-related factor 2
pMDI: Pressurized metered-dose inhaler
SOCS: Suppressor of cytokine stimulation
SRC-1: Steroid receptor coactivator 1
STAT: Signal transducer and activator of transcription
TCR: T-cell receptor
TTP: Tristetrapolin

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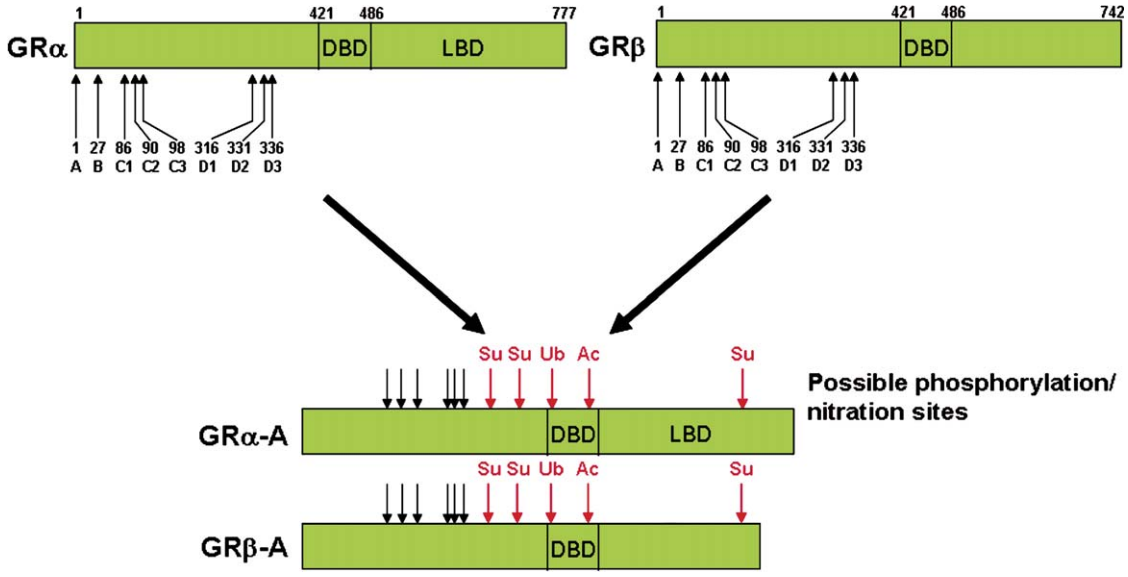

FIG 1. The GR can produce 8 distinct products (A, B, C1, C2, C3, D1, D2, and D3) by use of alternative translation initiation sites corresponding to methionine residues at 1, 27, 86, 90, 98, 316, 331, and 336. These alternative proteins can be produced from both GRα and GRβ transcripts. Posttranslational modification of GRs, particularly by means of phosphorylation and nitration, alters GR function and contributes to the potential for diverse function in distinct tissues. Other modifications, such as ubiquitination *(Ub)*, sumoylation *(Su)*, and acetylation *(Ac)*, are also shown.

well to glucocorticoid treatment, and these subjects account for approximately 50% of the total health care costs of asthma.[5,6] These subjects include patients with severe asthma, who are at increased risk of dying from asthma and who have continued morbidity from both their disease and the oral corticosteroids that are often used to treat it.[5,6] Furthermore, despite the availability of effective and relatively cheap treatments, there is still a considerable degree of undertreatment of severe asthma. For example, a European survey showed that only approximately 25% of patients with severe asthma were receiving inhaled corticosteroids.[7] In this review we will cover glucocorticoid receptor (GR) structure and function, limitations of glucocorticoid therapy, clinical characteristics of patients with severe treatment-insensitive asthma, and mechanisms underlying this insensitivity. Finally, we will discuss current treatment strategies and the potential for novel stand-alone or add-on therapies being developed that might be suitable for this group of subjects.

GLUCOCORTICOID ACTION

Structure of the GR and its gene

Glucocorticoids exert their effects by binding to a ubiquitously expressed 777-amino-acid GR that is localized to the cytoplasm of target cells. GR is a modular transcription factor in which specific domains play selective roles (Fig 1).[4] Although unliganded GR is thought to remain in the cytoplasm, evidence with nuclear export inhibitors suggests that a rapid active cycling of GR between the nucleus and cytoplasm might occur.[8,9] 2 GR isoforms (α and β) were originally described (Fig 2), with the nuclear GRβ having a dominant negative effect on GRα through the formation of GRα/GRβ heterodimers.

Yudt and Cidlowski[10] originally proposed the existence of additional isoforms of GR through use of different initiation sites within exon 2. Thus 4 distinct isoform of GR were proposed GRα-A, GRα-B, GRβ-A, and GRβ-B, depending on the methionine codon used and the GR N-terminus (Fig 1). Interestingly, GRα-B has twice the biologic activity of GR-αA *in vitro*, and this suggests that differential expression in various cell types might explain distinct cellular responsiveness.[10] To complicate things further, however, it has recently been reported that each single GR mRNA species can produce up to 8 functional GR N-terminal isoforms through leaky ribosomal scanning and shunting mechanisms (Fig 1).[11] These GR isoforms display diverse cytoplasm-to-nucleus trafficking patterns and distinct transcriptional activities. Importantly, in these studies the transcriptional responses to dexamethasone closely reflect the identity and abundance of the GR isoforms in human osteosarcoma cells. Advances in gene expression profiling techniques have allowed the effects of mutations in GR to be studied across a range of responsive genes in parallel. Such studies have revealed that different sets of genes are affected in different ways by each mutant.[12] For instance, some genes are dependent on the N-terminal AF-1 domain, whereas for others this activity is redundant. Because expression of some cofactor proteins, such as the peroxisome proliferator-activated receptor (PPAR) γ coactivator 1, is controlled both temporally and spatially,[13] the opportunity for selective modulation of glucocorticoid response through the manipulation of affinity for cofactors becomes apparent.

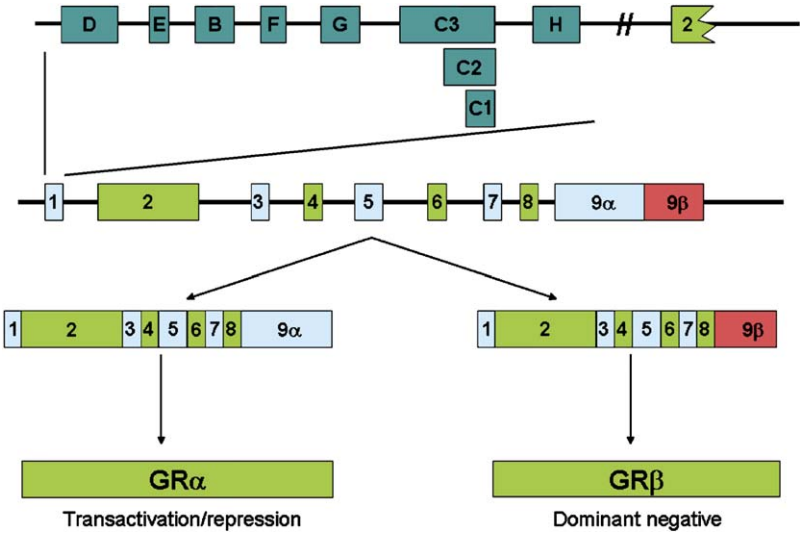

FIG 2. The GR is comprised of 9 exons. Alternative splicing of exon 9 at the 5′ end of the coding region leads to the formation of the classic GRα isoform and the dominant negative GRβ isoform. The multiple exon 1 variants that might control tissue-selective gene expression are also shown.

The GRγ splice variant is ubiquitously expressed at 5% of the levels of GRα and results from an alternate splice donor site in intron C of the GR gene (Fig 2). This results in the insertion of 3 extra nucleotides between exons 3 and 4, and therefore introduces an extra amino acid (arginine) at position 453 between the 2 zinc fingers of the GR DNA-binding domain.[14] GRγ is less transcriptionally active compared with GRα and as with GRβ can act as a dominant negative isoform.[14] Interestingly, patients with acute lymphoblastic leukemia who respond well to prednisolone therapy had reduced GRγ expression.[15,16] In contrast to GRβ and GRγ, the GR-P isoform, which has only 676 amino acids and is encoded by an mRNA encompassing part of intron 7 but lacking exons 8 and 9, is thought to enhance glucocorticoid activity.[17] These data suggest that different GR splice variants might affect glucocorticoid sensitivity by altering the transcriptional control of glucocorticoid-responsive genes.

GR mutations

Familial-sporadic glucocorticoid resistance syndrome is a GR-mediated disorder characterized by hypercortisolism without Cushingoid features and was first described in 1976.[18] Subsequently, several other kindreds and sporadic cases have been reported, showing abnormalities in either GR expression levels, affinity for glucocorticoids, stability, and/or translocation into the nucleus.[18] In most cases this is a result of specific mutations in the GR, such as D641V in the GR ligand binding domain (LBD); this mutation reduced binding affinity for dexamethasone by 3-fold and caused concomitant loss of transactivation activity.[19] Other mutations include V729I, I559N, I747M, and a 4-base deletion at the 3′ boundary of exon 6, removing a donor splice site and resulting in the complete loss of one of the GR alleles.[18] Because glucocorticoid-resistant asthma does not appear to involve GR mutations, such as those seen in familial glucocorticoid resistance syndrome, this review will not focus on these subjects or the effects of these mutations on GR function. Furthermore, modification of Y735 in GR selectively impairs transactivation without affecting transrepression through the differential recruitment of nuclear receptor corepressor (NCoR) 1 rather than steroid receptor coactivator 1 (SRC-1), allowing a molecular switch to occur.[20]

GR LBD structure

The crystal structure of the GR LBD has been determined in a ternary complex with dexamethasone and a transcription initiation factor (TIF) 2 coactivator peptide after point mutation of F602.[21] The overall structure is similar to that of other nuclear hormone receptor LBDs but contains a unique dimerization interface and a second charge clamp that might be important for cofactor selectivity. Unlike other nuclear hormone receptor LBDs, the GR LBD also has a distinct binding pocket opposite the C17 of dexamethasone that might explain ligand selectivity. In addition, similar to the results seen with the estrogen antagonist raloxifene binding to estrogen receptor, RU486 binding to the GR LBD results in a failure of helix 12 to correctly close over the binding cleft.[22] This results in a conformation more able to recruit corepressors than the closed helix 12, which efficiently recruits coactivators[22,23] and has led to rational-based design of selective dissociated GR agonists.[24] Modification of the dimerization interface by mutation of I628A resulted in reduced transactivation ability without affecting the ability to repress a nuclear factor κB (NF-κB)–driven reporter gene. The contrasting effects of this mutant suggest that the monomer and dimer forms of GR might regulate distinct signaling pathways, confirming data obtained from the *dim(−/−)* mouse.[25] Knowledge of the crystal structure of the GR LBD has led to the development of selective dissociated ligands.[24]

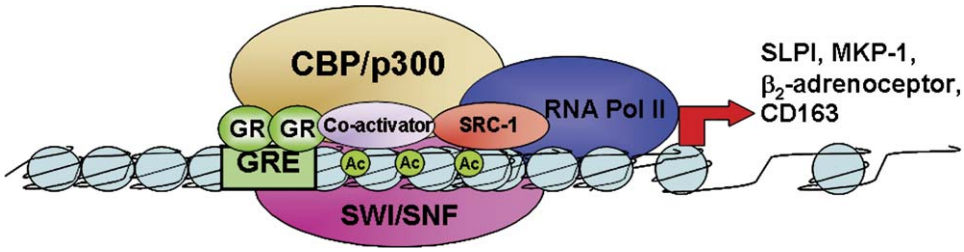

FIG 3. Mechanism of gene activation by the GR. Corticosteroids can freely diffuse across the plasma membrane, where they associate with the inactive cytosolic GR. On ligand binding, the GR is activated and can translocate to the nucleus, where it binds to a GRE within the controlling region for glucocorticoid-responsive target genes. These GREs might be 5′ or 3′ to the start site for transcription. Once bound to DNA, GRs recruit a complex containing basal transcription factors, coactivators (eg, CBP and SRC-1), and chromatin modifiers (eg, SWI/SNF and RNA polymerase II *[RNAP II]*), which together induce histone modifications, including acetylation *(Ac)* and chromatin remodeling, and subsequent production of mRNAs encoding various genes, including SLPI, MKP-1, CD163, and the β_2-adrenoceptor. *SLPI*, Secretary leukocyte protease inhibitor.

Gene induction by GR

Glucocorticoids are 21-carbon steroid hormones composed of 4 rings[26] that are thought to freely diffuse from the circulation into cells across the cell membrane and bind to cytoplasmic GR. Once the glucocorticoid binds to GR, hsp90 dissociates, allowing the nuclear localization of the activated GR-glucocorticoid complex and its binding to DNA. Nuclear importation of proteins is an active process for proteins larger than 40 kd that contain a nonconsensus basic targeting sequence or nuclear localization sequence (NLS).[27] GR contains a classical basic NLS (NLS1, residing in the hinge region) and a second, only poorly characterized NLS residing across the LBD,[28] and nuclear import through NLS1 proceeds more rapidly than that seen under NLS2 control.[28] Recent studies have shown that nuclear import of GR is mediated through its NLS and interaction with importins, with importin α selectively binding to NLS1[29] and importins 7 and 8 binding to both NLS1 and NLS2.[30] GR nuclear export is also tightly regulated; however, the role of the exportin 1 (chromosome maintenance region 1 [CRM-1]) pathway is currently unclear.[9,28] Importantly, the NLS–importin α interaction is often influenced directly by the phosphorylation status of the imported proteins.[27]

Within the nucleus, one GR can combine with another GR to form a dimer at consensus DNA sites termed glucocorticoid response elements (GREs; GGTACAnnn TGTTCT) in the regulating regions of corticosteroid-responsive genes (Fig 3). This interaction allows GR to associate with a complex of transcriptional coactivator proteins, including SRC-1 and cyclic AMP response element–binding protein (CREB)-binding protein (CBP), which produce a DNA-protein structure that allows enhanced gene transcription.[25] Recent evidence has suggested that individual GR ligands can target GR to specific nuclear subdomains,[31] and therefore the magnitude and direction of the transcriptional response to glucocorticoids is dependent on the particular ligand, the number of GREs, and the position of the GREs relative to the transcriptional start site.[32]

Many genes, including liver-specific metabolic genes, such as tyrosine aminotransferase, and stress response genes, such as metallothionein, contain clearly identifiable GREs in their promoter regions, allowing activated GR to bind to DNA as a homodimer, recruit transcriptional coactivators, and induce gene transcription.[4] In contrast, the expression of some genes, such as prolactin and osteocalcin, is decreased on GR-GRE binding. The number of genes per cell directly regulated by glucocorticoids is estimated to be between 10 and 100, but many genes are indirectly regulated through an interaction with other transcription factors and coactivators.[25] Recent array experiments in A549 lung epithelial cells using cyclohexamide to prevent secondary effects indicate that dexamethasone can directly upregulate 108 genes and downregulate 73 genes after 6 hours, many involved in proliferation, apoptosis, inflammation, and surfactant synthesis, all of which are primary glucocorticoid-controlled processes.[32] It seems highly unlikely that the widespread anti-inflammatory actions of glucocorticoids could be explained by increased transcription of small numbers of anti-inflammatory genes, such as annexin 1, IL-10, and the inhibitor of NF-κB (IκBα). However, therapeutic doses of inhaled glucocorticoids have not been shown to increase annexin 1 concentrations in bronchoalveolar lavage (BAL) fluid,[33] and an increase in IκBα has not been shown in all cell types.[34] In addition, it is likely that glucocorticoid side effects, such as osteoporosis, cataracts, growth retardation in children, skin fragility, and metabolic effects, are due to gene activation.[35]

Chromatin modifications and gene transcription

GRs, like other transcription factors, increases gene transcription through recruitment and activation of transcriptional coactivator proteins[4] (Fig 3) through LXXLL motifs.[36] Expression and repression of genes is associated with alterations in chromatin structure by means of enzymatic modification of core histones.[37] Specific residues (lysines, arginines, and serines) within the N-terminal tails of core histones are capable of being posttranslationally modified by means of acetylation, methylation, ubiquitination, or phosphorylation, all of which have been implicated in the regulation of gene expression.[37] The ''histone code'' refers to these modifications, which are

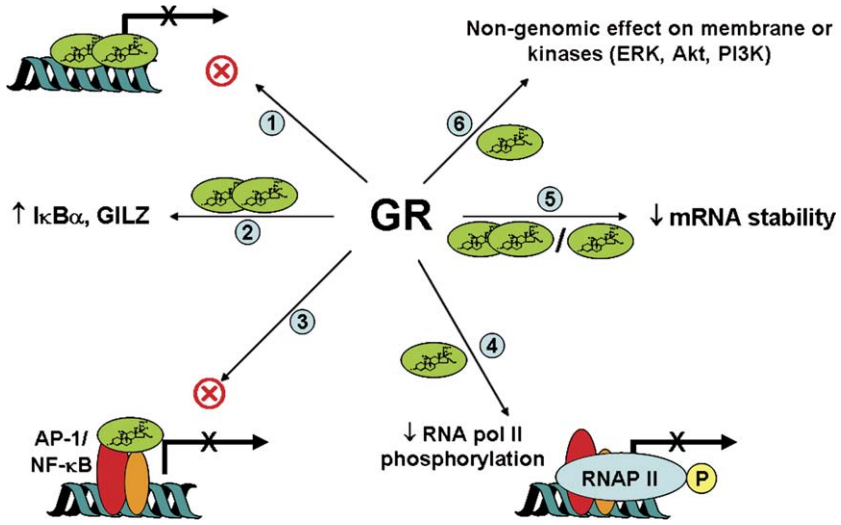

FIG 4. Mechanisms of gene repression by the GR. Activated GRs acting as homodimers can bind to GREs that overlap the DNA-binding site for a proinflammatory transcription factor or the start site of transcription to prevent inflammatory gene expression *(1)*. Activated GRs can repress AP-1/NF-κB–mediated gene expression by either inducing the expression of the NF-κB inhibitor IκBα or the AP-1 inhibitor glucocorticoid-inducible leucine zipper *(GILZ)* through classic gene induction mechanisms *(2)*. Acting as a monomer, GRs can directly or indirectly suppress AP-1/NF-κB activity by *(3)* altering cofactor activity or by *(4)* dephosphorylating RNA polymerase II *(RNA pol II)*. GRs can also reduce the levels of mRNA by either inducing the dual-specificity MAPK phosphatase 1 *(MPK-1)* that in turn regulates p38 MAPK-mediated mRNA stability or increasing the levels of cell ribonucleases and mRNA destabilizing proteins *(5)*. Finally, GRs can induce rapid nongenomic effects either through a membrane receptor or affecting activation of kinases, such as ERK, Akt, or PI3K *(6)*. Mechanisms that have been reported to be reduced under conditions of oxidative stress are shown as a circled "X". *PI3K,* Phosphatidylinositol 3-kinase.

set and maintained by histone-modifying enzymes and contribute to coactivator recruitment and subsequent increases in transcription.[38,39]

Transcriptional coactivators, such as CBP and p300/CBP-associated factor, have intrinsic histone acetyltransferase (HAT) activity,[37] and increased gene transcription is therefore associated with an increase in histone acetylation. In contrast, hypoacetylation induced by histone deacetylases (HDACs) is correlated with reduced transcription or gene silencing.[37]

GRs interact with CBP and other coactivator proteins, including SRC-1, TIF-2, p300/CBP cointegrator protein, and glucocorticoid receptor interacting protein 1 (GRIP-1), that enhance local HAT activity.[40,41] For example, dexamethasone at high concentrations ($\geq 10^{-8}$ M) in A549 cells enhances binding of activated GRs to CBP, associated coactivators, or both, resulting in histone acetylation on lysines 5 and 16 of histone H4 and increased gene transcription.[40] In addition, correct association of a GR with DNA and other proteins determines the assembly of coactivator complexes on GR target promoters, resulting in differential acetylation of histones on distinct lysine residues.[42] Nuclear hormone receptors do not in themselves recruit all the cofactors required at target promoters,[43] but steroid receptor coactivators, recruited by GRs, can in turn recruit other coactivators and chromatin remodeling complexes[41] that aid in the formation of the transcription initiation complex and result in local chromatin remodeling (Fig 3).[4]

In addition to the more classical modes of action, it is now clear that GRs can bind to DNA as heterodimers with other transcription factors, such as members of the signal transducer and activator of transcription (STAT) family[44,45] and the ETS transcription factors,[46] leading to the recruitment of distinct coactivator (eg, GRIP-1) or corepressor (eg, RIP140 or HDAC) complexes.[20,23,47] These interactions are exemplified by those seen with STAT5, whereby GR association with STAT5, independent of GRE DNA binding, enhances β-casein expression, but STAT5 overexpression leads to a reduction in GR-GRE action.[45,48] In addition, vitamin B6 can reduce dexamethasone-stimulated GRE activity without affecting transrepression.[49]

GENE REPRESSION BY GRs

In spite of the ability of glucocorticoids to induce gene transcription, the major anti-inflammatory effects of glucocorticoids are through repression of inflammatory and immune genes (Fig 4). Most glucocorticoid-repressible inflammatory genes do not posses negative GREs in their promoters, and the expression of some genes, such as prolactin and osteocalcin, are decreased on GR-GRE binding as a result of the GRE being positioned across a binding site for another transcription factor or across the transcriptional start site.[4] The inhibitory effect of glucocorticoids appears to be due largely to interaction between

the activated GR and the transcription factors, such as NF-κB and activator protein 1 (AP-1), which mediate the expression of inflammatory genes.[25] Full inflammatory gene expression probably requires that a number of transcription factors act together in a coordinated manner, and glucocorticoids, by targeting several transcription factors, might have greater effects than if only a single factor was targeted.[4] The precise mechanism for this repression is still unclear and might include binding to or recruiting nuclear receptor corepressors,[40,50] direct repression of coactivator complexes,[40,51] or effects on RNA polymerase II phosphorylation.[52,53] These effects are context-gene dependent, however, because many NF-κB–driven genes are not repressed by activated GRs, such as IκBα. Moreover, GRs can combine with NF-κB to induce the expression of Toll-like receptor 2 and stem cell factor.[54,55]

Recent evidence indicates that dexamethasone-activated GRs repress a large set of functionally related inflammatory genes stimulated by p65/interferon response factor 3 (IRF-3) complexes.[56] In contrast, PPARγ and liver X receptors repress overlapping transcriptional targets in a p65/IRF-3–independent manner and cooperate with GRs to suppress distinct subsets of pattern recognition receptor–responsive genes.[56] Designing drugs with the capacity to activate GRs and other nuclear hormone receptors might therefore enhance the anti-inflammatory profile of glucocorticoids. Moreover, because the expression of many cofactors and nuclear receptors is tissue specific, there is the attractive possibility of designing tissue-specific ligands, although this approach will require a clearer understanding of the key tissue or tissues that are targeted by glucocorticoids.

NF-κB

NF-κB is activated by numerous extracellular stimuli, including cytokines (eg, TNF-α and IL-1β), viruses, and immune challenges.[57] NF-κB is ubiquitously expressed within cells and is able to not only control induction of inflammatory genes in its own right but can enhance the activity of other cell- and signal-specific transcription factors.[58] Activation of the classic p65/p50 heterodimer by cell-surface receptors has been well described elsewhere[59] and involves phosphorylation of a specific kinase (inhibitor of NF-κB kinase [IKK]).[59] Subtle changes in p65 phosphorylation are also important; for example, inactive p65 is nonphosphorylated and is associated predominantly with HDAC1, whereas p65 is phosphorylated after IKK-2 stimulation and is able to bind to coactivator molecules, such as p300/CBP.[60] NF-κB, as with GRs, can induce histone acetylation and other histone modifications in a temporal manner,[40,61] leading to recruitment of other coactivator and remodeling complexes and the induction of inflammatory gene expression.

It has become apparent that NF-κB activated by distinct cellular stimuli can control the expression of different patterns of genes.[56,62,63] Thus LPS and TNF-α induced distinct IKK profiles with respect to the amplitude and duration of activation and rate of decay in murine cells. TNF-α–induced IKK activity was rapidly attenuated by negative feedback induction of IκBα,[64] whereas LPS signaling and LPS-specific gene expression programs were dependent on a cytokine-mediated positive feedback mechanism. Toll-like receptor 4 signaling to IKK is mediated through the adaptor protein MyD88 and also through a less well-described MyD88-independent pathway that uses TNF receptor–interacting factor and IRF-3 to induce TNF-α and subsequently IKK with delayed kinetics.

The results suggest that TNF-α, and possibly other proinflammatory cytokines induced by TNF-α, can induce a positive feedforward loop to stimulate IKK activity with an altered activation profile and subsequently induce a distinct gene profile. It is postulated that similar events might also occur in relation to other signaling pathways, such as the mitogen-activated protein kinases (MAPKs). Thus the distinct biologic responses to LPS and TNF-α depend on signaling pathway–specific mechanisms that regulate the temporal profile of IKK activity. Furthermore, it has also become clear that small changes in the consensus κB binding site and surrounding bases can have profound effects on the subsequent ability of activated NF-κB to activate gene expression.[52]

GR suppression of NF-κB and AP-1

GR dimerization–deficient mice[65,66] indicate that GRs, acting as monomers, can bind directly or indirectly with the transcription factors AP-1 and NF-κB (Fig 4). The interaction between AP-1/NF-κB and GR might result from distinct mechanisms (Fig 4), including GR binding to or recruiting nuclear receptor corepressors (eg, NCoR and HDACs),[40,56] effects on RNA polymerase II phosphorylation,[52,53] or possibly direct repression of NF-κB–associated HAT activity at the GM-CSF promoter in epithelial cells.[40] Similar data have also been reported in primary airway smooth muscle cells, where fluticasone was able to attenuate TNF-α–induced p65 association with the native CCL11 promoter and block TNF-α–induced histone H4 acetylation.[67] In addition, other epigenetic marks on histones have also been reported to be targets for dexamethasone action. Thus TNF-α–induced phosphorylation of Ser10 of histone H3, possibly caused by p38 MAPK,[68] is rapidly inhibited and redistributed away from sites of active gene transcription in a time- and concentration-dependent manner by means of dexamethasone in BEAS-2B cells.[69]

Other mechanisms for GR suppression of AP-1 and NF-κB have been proposed (Fig 4). The GR dimer can induce the expression of the NF-κB inhibitor IκBα in certain cell types.[34,70] Similarly, induction of glucocorticoid-inducible leucine zipper can prevent AP-1 DNA binding and activity in some cells.[71] In addition, glucocorticoids might play a role in repressing the action of MAPKs, such as the extracellular signal–regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK).[4] These actions are mutually inhibitory[72,73] and, in the case of p38 and JNK, might relate to induction of the dual-specificity MAPK phosphatase 1 (MKP-1), which thereby attenuates MAPK activation.[74] Furthermore, we and others

have shown that p38 MAPK–mediated GR phosphorylation can attenuate GR function.[75,76]

Regulation of mRNA stability

Glucocorticoids also appear to exert anti-inflammatory actions that do not depend on the receptor's ability to regulate transcription in the nucleus (Fig 4). Adenylate-uridylate–rich elements (AREs) are found within the 3′ untranslated region of many inflammatory genes and control the stability of mRNA.[77,78] These sequences are very heterogeneous and include both AUUUA pentamers and AT-rich stretches. Binding of mRNA to ARE-binding proteins results in the formation of messenger ribonucleoprotein complexes, which control mRNA decay.[77,78] Several ARE-binding proteins have been reported and include tristetrapolin (TTP), which promotes mRNA decay, and Hu antigen R (HuR) family members, which are associated with mRNA stability. Importantly, HuR binding to AREs is dependent on p38 MAPK.[77,78] Dexamethasone has been reported to regulate the levels of HuR and TTP, thereby reducing the levels of inflammatory gene mRNAs, such as COX-2 and CCL11,[12,79] through a p38 MAPK–mediated pathway subsequent to induction of MKP-1.[80,81] However, significant modulation of these genes often appears at 10 nM dexamethasone rather than the 1 nM concentrations associated with suppression of many inflammatory genes, although again these effects might be cell selective.[82] Intriguingly, dexamethasone has recently been reported to decrease TTP expression in LPS-stimulated murine macrophages.[82]

Nongenomic actions of glucocorticoids

The traditional genomic theory of steroid action, whether directly interacting with DNA or involving cross-talk with other transcription factors, does not fully explain the rapid effects of hormonal steroids (Fig 4), and it is thought that the nongenomic actions are mediated by a distinct membrane receptor.[83,84] Initially described in amphibians, these receptors have been described in mammalian cells and have distinctive hormone-binding properties compared with the well-characterized cytoplasmic receptor and are probably linked to a number of intracellular signaling pathways acting through G-protein–coupled receptors and a number of kinase pathways.[83,85,86] An important effect seen in asthma that occurs through this mechanism is change in bronchial blood flow induced by inhaled corticosteroids.[87] In addition, the classical receptor is associated with a number of kinases and phosphatases within the inactive GR-hsp90 complex.[88] These enzymes are released on hormone binding and might also account for the rapid induction of tyrosine kinases seen in some cell types by glucocorticoids.[89,90]

Posttranslational modifications of GR

GR is a phosphoprotein containing numerous potential phosphorylation sites, including those for ERK, p38 MAPK, protein kinase C, and protein kinase A. Evidence obtained during the past 10 years clearly suggests that altered GR phosphorylation status can affect GR-ligand binding,[75] hsp90 interactions,[91] subcellular localization,[92,93] nuclear-cytoplasmic shuttling,[94,95] and transactivation potential,[92] possibly through association with coactivator molecules (Fig 1).[93]

Ligand binding induces GR hyperphosphorylation at 7 sites that regulate transactivation and reduces nonspecific DNA binding,[96] although this response varies during the cell cycle, with cells being less sensitive to corticosteroids during G2/M.[97] Thus global changes in GRs charge might affect their function, as well as specific phosphorylation events. MAPK activation or overexpression can also target specific serine-threonine residues in GRs, decreasing GR-mediated transactivation,[75,76] possibly through an effect on Ser226 phosphorylation and increasing GR nuclear export.[98] In addition, recent evidence suggests that GR phosphorylation is involved in receptor turnover and that phosphorylation can target the receptor for hormone-mediated degradation.[99] As such, phosphorylation-induced targeting of GRs for ubiquitination and proteosomal degradation might play an important role in overall GR responsiveness. GR sumoylation appears to have the opposite effect of ubiquitination and results in increased GR activity,[100] possibly through changes in cofactor recruitment (Fig 1).[101]

In addition to phosphorylation, nitrosylation of GR at an hsp90 interaction site induced by the nitric oxide (NO) donor S-nitroso-DL-penicillamine has also been shown to prevent GR dissociation from the hsp90 complex and a reduction in ligand binding.[102] It has become clear that histones are not the only targets for histone acetylases, and recent evidence has suggested that acetylation of transcription factors can modify their activity. For example, the p65 component of NF-κB can also be acetylated, thus modifying its transcriptional activity.[103] Recent evidence suggests that GR is also acetylated (Fig 1) on ligand binding and that deacetylation is critical for interaction with p65, at least at low dexamethasone concentrations.[104]

GLUCOCORTICOID RESISTANCE IN ASTHMA

Although glucocorticoids 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 even to high doses of oral glucocorticoids.[105] Resistance to the therapeutic effects of glucocorticoids is also recognized in other inflammatory and immune diseases, including rheumatoid arthritis and inflammatory bowel disease.[106,107] Glucocorticoid-resistant patients, although uncommon, present considerable management problems. It is likely that there is a spectrum of glucocorticoid responsiveness, with the rare resistance at one end, but a relative resistance is seen in patients who require high doses of inhaled and oral glucocorticoids (glucocorticoid-dependent asthma).[108]

Limitations of glucocorticoid therapy

Glucocorticoid resistance in asthma is not absolute, and patients often respond to very high doses of inhaled

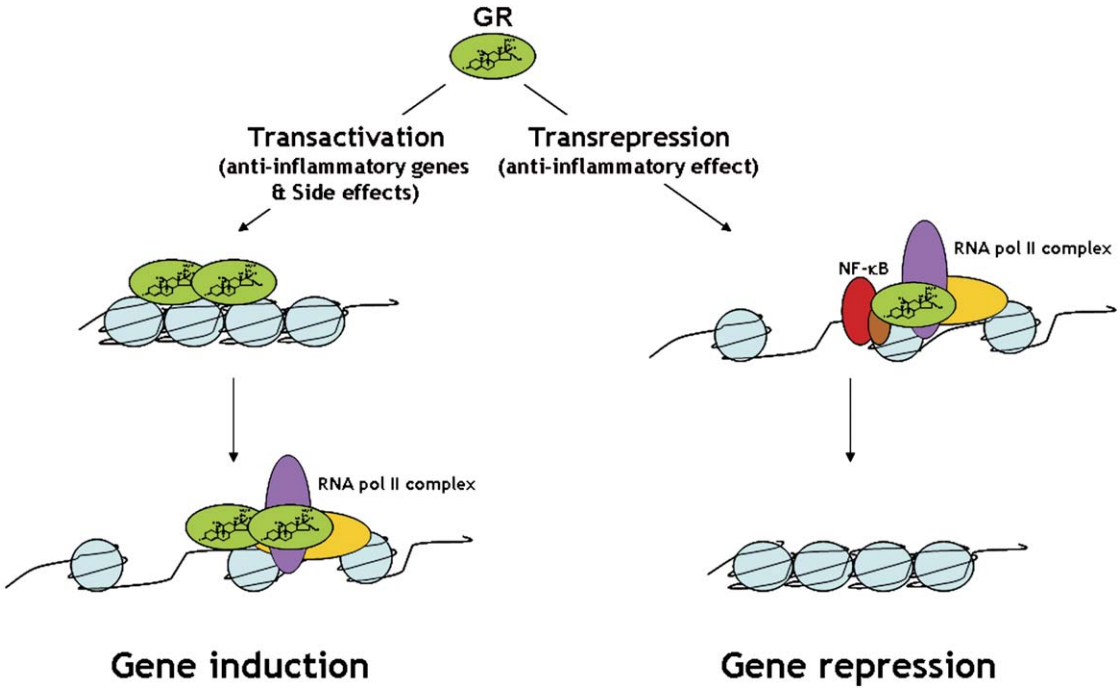

FIG 5. Rationale for dissociated glucocorticoids. Most anti-inflammatory actions of glucocorticoids are mediated through the GR monomer interacting with proinflammatory transcription factors, such as AP-1 and NF-κB, which activate gene expression by reversing the active state of chromatin. In contrast, gene-induction events mediated by a GR homodimer are responsible for many of the detrimental side effects of glucocorticoids, as well as the induction of some anti-inflammatory genes. *RNA pol II*, RNA polymerase II.

and/or oral glucocorticoids. However, the debilitating side effects of high-dose glucocorticoid therapy militate against its use in ever-increasing doses. All currently available topical glucocorticoids are absorbed into the systemic circulation and therefore inevitably have some systemic effect, although this is considerably less than that seen with oral glucocorticoids.[35] The occurrence and severity of the side effects seen depend on the duration of use, dosage, dosing regimen, and specific drug used, along with individual patient variability.[35] However, the highest risk factor appears to be prolonged use. Side effects of topical glucocorticoids include glaucoma, cataracts, tissue atrophy, and wound healing, whereas at high doses there is an increased risk of infection, adrenal suppression, and osteoporosis. The growth retardation seen with oral glucocorticoids does not appear to be a problem with modern topical glucocorticoids, although there might be an initial reduction in growth velocity on starting therapy. Side effects of oral glucocorticoids include skin and muscle atrophy, delayed wound healing and increased risk of infection, osteoporosis and bone necrosis, glaucoma and cataracts, behavioral changes, hypertension, peptic ulcers and gastrointestinal bleeding, Cushing's syndrome, and diabetes.[35] Interestingly, it appears that early skin atrophy induced by glucocorticoid therapy is reversible, whereas major atrophy leading to striae formation is not.[109]

These side effects often occur together, and this is exemplified by Cushing's syndrome, the signs and symptoms of which include increased blood pressure, development of diabetes, pink-to-purple stretch marks on the abdominal skin, fatigue, depression, moodiness, and accentuated fatty tissue on the face and upper back (Buffalo hump).[35,110] Women with Cushing's syndrome often have irregular menstrual periods and experience new facial hair growth. Men can show a decrease in sex drive. Taken together, these side effects seriously limit the value of glucocorticoids in patients with severe inflammation that fails to respond to moderate-high doses where the risk/benefit ratio is compromised. This has driven the need to develop novel agents with the anti-inflammatory capacity of corticosteroids but with reduced side effects.

Although the major anti-inflammatory effects of glucocorticoids are almost certainly a result of transrepression, the underlying molecular mechanisms for the side effects of glucocorticoids are complex and not fully understood.[35] Certain side effects, such as diabetes and glaucoma, are due to transactivation events, whereas others are caused by transrepression (hypothalamic-pituitary-adrenal axis suppression). In addition, the precise molecular events underlying glucocorticoid induction of osteoporosis are unclear but probably require both gene induction and gene repression.[35]

Despite this uncertainty, there has been a search for dissociated glucocorticoids that selectively transrepress without significant transactivation, thus potentially reducing the risk of systemic side effects (Fig 5). Several nonsteroidal selective GR agonists have recently been

reported that show dissociated properties in human cells and are now in clinical development, where they show good separation between transrepression and transactivation actions in the skin.[4,111,112] This suggests that the development of dissociated glucocorticoids with a greater margin of safety is possible and might even lead to the development of oral compounds that have reduced adverse effects. Furthermore, the newer topical glucocorticoids used today, such as fluticasone, mometasone, and budesonide, appear to have more potent transrepression than transactivation effects, which might account, at least in part, for their selection as potent anti-inflammatory agents.[4] These new potent glucocorticoids are particularly effective as topical agents, and their use has overtaken that of oral-systemic glucocorticoids for many diseases. For example, coated enteric slow-release budesonide capsules are equally effective as prednisolone in Crohn's disease, without the associated reduction in plasma cortisol seen with prednisolone.[113,114] Similar results have been achieved with fluticasone, albeit in fewer well-controlled studies.[115]

An alternative approach to obtain safer drugs is the use of soft drugs, such as ciclesonide, which are only activated at the site of inflammation. Ciclesonide itself is inactive and needs to be cleaved by lung-specific esterases to bind to GRs.[116] Ciclesonide had significantly less effect on serum cortisol levels than beclomethasone dipropionate,[116] suggesting that ciclesonide might have less systemic effects and hence a superior safety profile.

Severe treatment-insensitive asthma

In the study of severe glucocorticoid-insensitive asthma, it is essential that the patients are well characterized according to guidelines[117] and are taking their treatments and that confounding factors are removed.[118,119] This is essential because despite the availability of effective therapies, there is clear evidence for suboptimal asthma control in many patients worldwide, with long-term management falling far short of the goals set in the Global Initiative for Asthma guidelines.[120] Incorrect diagnosis, nonadherence with therapy, and psychiatric comorbidity might all contribute to difficulty in controlling asthma.[5,121] Recent studies from the Brompton Hospital focused on patients with severe asthma whose symptoms were not well controlled despite high doses of inhaled corticosteroids and other regular therapy.[122,123] Patients with severe asthma and irreversible airflow obstruction had longer disease duration, a greater inflammatory process as measured by exhaled NO and peripheral blood eosinophilia, and more high-resolution computed tomography airway abnormalities suggestive of airway remodeling, despite receiving similar treatments and experiencing equivalent impairment in quality of life.[122] Confirmation that increased levels of exhaled NO might be associated with high tissue and BAL fluid eosinophilia was obtained in a study of 24 patients with severe corticosteroid-resistant (CR) asthma in Denver.[124] However, the concept that high eosinophilia is refractive to therapy and is associated with a distinct phenotype has been recently challenged.[125] In subjects with severe CR asthma with high sputum eosinophilia despite high-dose (>1600 μg/d) inhaled glucocorticoids, 120 mg of triamcinolone was able to abolish sputum eosinophil counts and restore lung function in more than 80% of subjects after 2 weeks.[125] This suggests that eosinophil-directed treatments either used as a monotherapy or, more likely, as a steroid-sparing therapy might be useful for these subjects.

In addition, these studies also suggested that even in those patients referred to a tertiary center for assessment, there is a considerable rate of alternative or additional diagnoses (32%), nonadherence with therapy (approximately 50% patients receiving >15 mg/d prednisolone), and psychiatric comorbidity (11%).[123] These incidences might vary in primary care practice or in primary referral centers compared with those in a tertiary referral center, but they are unlikely to be lower.[126] Heaney et al[127] also found a high prevalence of comorbidity in patients with poorly controlled asthma but no difference in prevalence between those who responded to steroids and those who did not. Importantly, targeted treatment of identified comorbidities had minimal effect on asthma-related quality of life in those with glucocorticoid-insensitive disease.[127]

One potential reason for failure to respond to glucocorticoids in a small proportion of patients with severe asthma was the presence of CR asthma.[4,118] This was not formally assessed in this study, although a similar protocol looking at difficult asthma in children did suggest that a subgroup of patients had glucocorticoid insensitivity. CR asthma has been defined as a failure of the FEV_1 to improve from a baseline value of 75% of predicted value or less by 15% or more after 14 days of treatment with 40 mg of prednisolone taken orally, despite demonstrating greater than 15% reversibility to an inhaled β_2-agonist.[128] As stated above, there is a spectrum of steroid responsiveness, and both patients with corticosteroid-dependent (CD) asthma and patients with CR asthma present considerable management problems.[4,118] Importantly for examining the molecular basis of glucocorticoid insensitivity, CR asthma is also associated with impaired *in vitro* and *in vivo* responsiveness of PBMCs to the suppressive effects of glucocorticoids. Thus in patients with CR and CD asthma, there is a reduction in the inhibitory effect of glucocorticoids on cytokine release in PBMCs.[4,118]

Previous descriptions of severe or difficult-to-treat asthma using bronchoscopic studies and noninvasive measures of inflammation have suggested clinical subgroups, including brittle asthma and corticosteroid-insensitive asthma, and in some cases such classification might be a useful guide to management.[129,130] Success with other treatment regimens, including cyclosporin, methotrexate, FK506, and rapamycin, is variable, which might reflect the heterogeneity of disease.[131,132]

Similar results have been reported in a cross-sectional study of 163 patients with severe asthma throughout Europe (the European Network for Understanding Mechanisms of Severe Asthma study).[133] Overall, these patients with severe asthma were predominantly female, were

more aspirin sensitive, and had lower levels of atopy than patients with mild-to-moderate asthma. In addition, these subjects had greater airway obstruction, increased air trapping, and a slightly lower diffusing capacity. Sputum eosinophil numbers remained increased despite high-dose inhaled and often oral steroids (30% of subjects), and importantly, there was a marked increase in sputum neutrophilia. These studies again suggest that severe asthma might be a separate disease from mild-to-moderate, therapy-responsive asthma.[133]

Previous studies have shown that sputum and tissue eosinophilia varies in patients with severe asthma, with a subgroup showing normal levels and the other showing increased levels. This subset of patients with high eosinophil counts has been shown previously to have a greatly thickened basement membrane, suggesting a difference in airway remodeling.[134] Recent data from Flood-Page et al[135] give further evidence for a role of the eosinophil in inducing changes in airway remodeling. This difference might account for the profound differences seen in reversibility and other measures of lung function in some patients with severe asthma. However, there are no biologic-disease markers that clearly differentiate one group with severe asthma from the other, and current markers overlap.

In addition to reduced changes in clinical symptoms after corticosteroid therapy, studies have shown that there is reduced suppression of IL-4 and IL-5 mRNA in BAL cells obtained from patients with CR asthma after 1 week of treatment with prednisolone when compared with those of patients with corticosteroid-sensitive (CS) asthma.[136] BAL of a group of patients with CR asthma revealed an increased number of cells expressing IL-2, IL-4, and IL-13 mRNA compared with those seen in patients with steroid-sensitive asthma.[136] This suggested that the profile of cytokine expression might underlie the poor responsiveness to glucocorticoids in these patients. Whether these changes in mediator expression relate to the report that alveolar macrophages from patients with CR asthma are less able to phagocytose T cells after LPS stimulation is unclear.[137] Interestingly, this study also showed that the defect in phagocytosis was overcome with very high concentrations of dexamethasone *in vitro*, although the effects of more moderate concentrations of dexamethasone were not reported.

It is currently unclear as to why these patients might respond less well to inhaled and oral glucocorticoids. A distinct pathophysiology present in the population with severe asthma might account in part for these differences in responsiveness (eg, neutrophilic inflammation[134] or in some cases a T-cell and eosinophillic inflammation[124,138]), but other explanations might involve induction of glucocorticoid insensitivity that prevents glucocorticoids from functioning effectively on the same pathologic processes that occur in mild asthma[4,118] or that excessively remodeled airways are fixed and nonresponsive to corticosteroids.[139,140]

Further work is required to correlate clinical and inflammatory phenotypes of asthma with treatment response. The European Network for Understanding Mechanisms of Severe Asthma data suggest that rather than severe asthma being a distinct disease, it might consist of several different diseases. Ongoing studies, such as the European Bio-Air study, which will include biopsy data, and the US Epidemiology and Natural History of Asthma: Outcomes and Treatment Regimens study might provide an answer to this question.[130]

MOLECULAR MECHANISMS OF CORTICOSTEROID RESISTANCE

At a molecular level, resistance to the anti-inflammatory effects of glucocorticoids can be induced by several mechanisms, and these might differ between patients. The reduction in corticosteroid responsiveness observed in cells from these subjects has been ascribed to a reduced number of GRs, altered affinity of the ligand for GRs, reduced ability of the GRs to bind to DNA, or increased expression of inflammatory transcription factors, such as AP-1, that compete for DNA binding.[108,118]

Defects in GR sequence and pharmacokinetics

Unlike familial corticosteroid resistance, in which there is a mutation in the LBD of the GR and a subsequent resetting of the basal cortisol level, patients with CR asthma have normal cortisol levels and are not Addisonian.[141] By using standard dexamethasone suppression tests, it has been shown that patients with CR asthma do not have an altered secretory rate of endogenous cortisol or an altered sensitivity of the hypothalamic-pituitary-adrenal axis.[142] By using chemical mutational analysis, no mutations in the GRs of patients with CR asthma were observed.[143] This was confirmed in a later study that used RT-PCR.[144] Therefore it is unlikely that the defect in CR asthma lies in the structure of the GR.

Defects in ligand binding

Certain cytokines (particularly IL-2, IL-4, and IL-13, which are overexpressed in bronchial biopsy specimens of patients with steroid-resistant asthma) might 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.[105,145]

Initial studies with whole cell binding assays failed to demonstrate any significant changes in monocyte and T-cell binding affinity (dissociation constant [Kd]) and receptor density of the GR in patients with CR asthma.[146-148] However, using analysis of GR in subcellular compartments, Sher et al[149] were able to describe 2 patterns of ligand-binding abnormalities in patients with CR asthma termed types 1 and 2. The more common type 1 defect was associated with reduced Kd of the GR and normal receptor numbers and was specific to T cells. The less common type 2 defect was associated with reduced GR receptor density with a normal Kd and was seen in the total mononuclear cell population. The type 1 defect

was reversible with serum deprivation and was mimicked by incubation of cells with high concentrations of IL-2 and IL-4 or by IL-13 alone.[75,118,149] In contrast, the type 2 defect was irreversible and was not IL-2 and IL-4 dependent.[149] These differences were detected only in the nucleus and not in the cytoplasm, possibly reflecting an effect of a nuclear protein masking the GR ligand-binding site or in an altered conformation of the activated GR. Differences in Kd in CR asthma have been confirmed in other cohorts of patients.[75,150] Interestingly, in one of these studies, changes in Kd were not correlated with changes in airway remodeling.[150] This altered affinity of dexamethasone for the GR might reflect either an intrinsic defect in the GR within these patients or might relate to changes in the receptor induced by the type of inflammation seen in patients with CR asthma. The reversal of the reduced binding affinity by means of incubation with normal media suggests that the latter is a more likely possibility.[75,118]

Two explanations for the effect of IL-2 and IL-4 or IL-13 alone on ligand-binding characteristics have been proposed. Leung and Bloom[118] have associated these changes in ligand-binding characteristics seen in peripheral blood cells with an increased expression of the dominant negative isoform of GR, GRβ,[118] although others have been unable to detect enhanced GRβ expression in PBMCs from other cohorts of patients with CR.[75,151,152] Interestingly, the highest expression of GRβ is seen in neutrophils, which are relatively corticosteroid insensitive.[153] In contrast, increased numbers of cells expressing GRβ have been reported in skin biopsy specimens from patients with CR asthma.[154] More conclusively, recent evidence in BAL fluid macrophages obtained from patients with CR asthma shows increased expression of GRβ mRNA and staining.[155]

We have recently demonstrated that the effects of IL-2 and IL-4 and those of IL-13 on GR ligand binding and dexamethasone regulation of IL-10 release were blocked by the p38 MAPK inhibitor SB203580.[75] Activation of p38 MAPK by IL-2 and IL-4 resulted in serine phosphorylation of the GR and reduced dexamethasone repression of LPS-stimulated GM-CSF release. The ability of dexamethasone to modulate IL-10 release was also inhibited by IL-2 and IL-4 cotreatment and restored by SB203580.[75] It is unclear whether this is a direct or indirect effect of p38 MAPK or whether GR phosphorylation alters ligand-binding affinity directly. This might result from either a change in GR conformation caused by association of distinct cofactors or partial blocking of the ligand binding domain caused by association of GR with nuclear transcriptional modulating proteins.

Similar results have been seen after NO treatment of the GR, whereby nitrosylation of the GR at an hsp90 interaction site modified ligand binding.[102] Serine 226 and the sequences immediately surrounding it are highly conserved, suggesting that its phosphorylation might alter or disrupt the protein-protein interactions regulating GR action. Interestingly, in Crohn's disease steroid resistance is associated with increased epithelial activation of JNK, p38 MAPK, NF-κB, and AP-1,[156] suggesting that drugs targeted toward these mediators might be useful in CR asthma. These data show that p38 MAPK inhibitors might have potential in reversing glucocorticoid insensitivity and reestablishing the beneficial effects of glucocorticoids in patients with severe asthma.

GR nuclear translocation and GR-GRE binding

We have previously reported that one subgroup of patients with CR and CD asthma exhibited impaired nuclear localization of the GR in response to a high concentration (10^{-6} M) of dexamethasone.[157] The mechanism for this effect is unclear but might reflect changes in GR phosphorylation by MAPK and subsequent interaction with importin α.[29,73,75] This might also explain the earlier results we obtained with electrophoretic mobility shift assays, which showed that patients with CR asthma had a reduced level of GR-GRE binding compared with that seen in patients with CS and nonasthmatic individuals after stimulation of PBMCs with dexamethasone.[158] Changes in GR-GRE binding have also been associated with excessive activation of AP-1, increased c-Fos expression, and JNK activity in response to inflammatory stimuli, such as TNF-α.[108,118] Scatchard analysis of GR-GRE binding showed no change in binding affinity but did show a reduced number of GRs available for DNA binding in the patients with CR asthma,[158] perhaps as a result of this reduction in GR nuclear translocation.[157] Therefore drugs that enhance GR nuclear translocation are likely to be of benefit in 50% of these patients.[88]

However, in another group of patients, nuclear localization of GRs is normal, and there is a defect in acetylation of histone 4.[157] In this group of patients, specific acetylation of lysine 5 is defective, and presumably this means that glucocorticoids are not able to activate certain genes that are critical to their anti-inflammatory actions.[157] This suggests that corticosteroids are not able to activate certain genes that are critical to the anti-inflammatory action of high doses of corticosteroids. We previously demonstrated an important role of p38 MAPK in the reduced actions of glucocorticoids in CR asthma. One important enzyme that is rapidly induced by GRs is MKP-1,[159] which dephosphorylates and inactivates p38 MAPK. Thus changes in p38/MKP-1 homeostasis might be important in contributing to steroid insensitivity.[160] The mechanism for this effect is unknown but might reflect the mutual inhibitory effects of excess MAPK, particularly JNK activation, which might affect GR nuclear export,[98] GR-mediated transcription responses,[73] or both through a failure of GRs to recruit specific coactivators.

An important study from Leung's group demonstrated that that incubation with IL-2 induced steroid insensitivity in the murine cell line (HT-2) as a result of an inability of the GR to translocate into the nucleus after dexamethasone treatment. This lack of steroid sensitivity was lost when cells were pretreated with a Janus-associated kinase (JAK) 3 inhibitor. Immunoprecipitation experiments revealed that phosphorylated STAT5 and the GR formed immune complexes, thus preventing GR nuclear import.

Persuasively, IL-2 could not induce dexamethasone insensitivity in splenocytes from STAT5 knockout mice.[161] These data suggest that JAK3 inhibitors, such as WHI-P131 (Parker-Hughes Institute), which inhibits degranulation and cytokine release from IgE receptor/FcεRI cross-linked mast cells and also shows immunosuppressive activity in the context of an animal model of autoimmune disease,[162] might be an effective drug for severe CR asthma. Interestingly, suppressor of cytokine stimulation (SOCS), a natural regulator of the JAK-STAT pathway, has been proposed to play a role in severe treatment-insensitive allergic disease.[163] Interestingly, IL-4 induction of SOCS3 is blocked by inhibitors of both the JNK and p38 MAPK pathways linking MAPK activation to SOCS induction.[164] It is important to confirm these studies in human cells because there are distinct differences between human and rodent GRs.[165]

Cross-talk with AP-1

We originally reported an increase in the basal levels of AP-1 DNA binding in the nuclei of PBMCs isolated from patients with CR asthma. In addition, there was a reduced ability of GRs to interact and repress AP-1, but not NF-κB, activity.[144] Furthermore, we have recently demonstrated enhanced c-Fos expression in bronchial biopsy specimens of patients with CR asthma.[108] These results suggested that AP-1 levels are altered in patients with CR asthma and that increased levels of AP-1 might prevent GR function. In a subsequent study with nuclear run-on, RT-PCR, and Western blotting, we demonstrated a 2- to 4-fold greater increase in the c-*fos* transcription rate and mRNA and protein expression in PBMCs isolated from patients with CR asthma compared with values in patients with CS asthma and healthy subjects.[166] Overexpression of c-Fos attenuated the ability of cells from patients with CS asthma to induce GR-GRE binding after 1 hour of dexamethasone treatment, and incubation of PBMCs from patients with CR asthma with antisense oligonucleotides directed against c-*fos* increased GR-GRE binding to similar levels seen in individuals with CS asthma. These findings suggested that increased c-Fos levels under basal conditions are the predominant inhibitory activity on GR DNA binding in CR asthma.

Using the tuberculin skin response as a model of mononuclear cell inflammation, Sousa et al[167] subsequently showed a marked increase in the expression of activated phosphorylated c-Jun, enhanced expression of JNK, and greater upregulation of c-Fos expression in the CR compared with the CS group. In this model prednisolone reduced the levels of both phosphorylated c-Jun and phosphorylated JNK in the CS group, but not the CR group, without affecting total c-Jun and JNK expression. More recently, c-fos, but not c-jun or GRβ, expression has been reported to inversely correlate with steroid sensitivity of PBMCs from asthmatic patients.[168]

The data to date suggest that increased levels of c-Fos and increased activation of c-Jun in patients with CR asthma accounts for the increased AP-1 activity seen *in vitro* and probably relates to increased activation of JNK in these subjects. JNK regulates the expression and activation of both major components of AP-1.[169] Therefore increased JNK activity could be critical to the mechanisms of CR asthma, and failure to inhibit JNK phosphorylation by glucocorticoids might be a major cause for the lack of response to glucocorticoids in CR asthma.[108] In addition, JNK might in turn suppress GR function,[73,98] resulting in a feedforward loop of increasing inflammation and reduced corticosteroid responsiveness in these patients. It is unclear whether increased c-*fos* transcription and JNK activation is a primary or secondary defect caused by excessive production of a unique pattern of cytokines in asthmatic airways. At present, there is no evidence for a genetic component leading to enhanced AP-1 activation in CR asthma. The increased numbers of BAL cells expressing IL-2 and IL-4 in the CR asthma group might suggest a primary defect of cytokine regulation in these patients. T_H2 cytokines can enhance AP-1 expression,[170] which in turn can switch on more T_H2 cytokines,[171] leading to a proinflammatory amplification loop. Irrespective of whether enhanced expression of AP-1 is primary or secondary, the net result is an excessive accumulation of this critical transcription factor.

OTHER FACTORS CONTRIBUTING TO GLUCOCORTICOID RESISTANCE

Immunomodulation

T_H2 cytokines have also been proposed to play a role in severe CR asthma. A recent study has shown that $CD4^+$ T cells from patients with CR asthma are less able to produce the anti-inflammatory cytokine IL-10 in response to dexamethasone than cells from patients with CS asthma.[172] These data suggest that therapeutic administration of IL-10 or of high IL-10–producing T regulatory cells might be effective in CR asthma. Furthermore, the same group has reported that addition of vitamin D3 in combination with dexamethasone can restore the ability of IL-10–producing $CD4^+$ T cells from patients with CR asthma to release IL-10 at similar levels to those seen in cells from patients with CS asthma.[173] This allowed IL-10 to upregulate GR expression in $CD4^+$ T cells. In addition, vitamin D3 reversed the dexamethasone-induced reduction in GR expression in these cells. Impressively, oral administration of vitamin D3 (0.5 μg/d) for 7 days to patients with CR asthma enhanced *ex vivo* T-cell responses to dexamethasone.[173] This suggests that vitamin D3 could potentially increase the therapeutic response to glucocorticoids in patients with CR asthma.

In addition, in a mouse model of asthma, IL-13 alone dose-dependently induced airway hyperresponsiveness (AHR), eotaxin release, eosinophilia, MUC5AC expression, and goblet cell hyperplasia.[174] Dexamethasone treatment attenuated CCL11 expression and completely abolished eosinophilia but had no effect on AHR, MUC5AC overexpression, or goblet cell hyperplasia. These data suggest that control of steroid-resistant features induced by IL-13, including AHR and mucus production, might provide new therapeutic modalities for asthma.[174]

The potent immunosuppressive properties of glucocorticoids are modulated by the conditions prevailing in the local immune milieu. This is exemplified by a recent study by Tsitoura and Rothman,[175] who showed that although dexamethasone was efficient at suppressing T-cell receptor (TCR)–induced $CD4^+$ T-cell proliferation that involves activation of c-Fos, AP-1, nuclear factor of activated T cells (NF-AT), and NF-κB, this ability was lost when cells were activated by TCR and CD28 costimulation. Interestingly, the inhibitory effects of dexamethasone against CD28-stimulated NF-AT and NF-κB were intact, as was the IL-2–stimulated STAT5 pathway, but the ability to suppress c-Fos/AP-1 activity was reduced.[175] Inhibition of the ERK pathway abolished the costimulation-induced resistance to dexamethasone.[175] These findings further suggest that MAPK inhibitors might offer a therapeutic solution for glucocorticoid resistance.

The cytokine milieu can also affect the ability of glucocorticoids to affect CD38 expression in primary human airway smooth muscle cells.[176] Thus CD38 induction stimulated by TNF-α, IL-1β, and IL-13 is sensitive to suppression by dexamethasone, budesonide, and fluticasone, but TNF-α and IFN-γ costimulation– or solely IFN-γ stimulation–induced CD38 expression was insensitive to glucocorticoid actions. This appeared to be due to induction of GRβ increasing the GRα/GRβ ratio to 1:3.[176]

Microarray studies in human bronchial epithelial cells indicate that dexamethasone is not very effective at suppressing IFN-γ–stimulated gene expression at 8 hours (2/66 genes) and 24 hours (45/287 genes).[177] Furthermore, many of the genes suppressed by dexamethasone at later time points were secondary, not primary, genes stimulated by proteins upregulated by IFN-γ. This inability of dexamethasone to affect IFN-γ–stimulated functions might be both temporal and cell specific because dexamethasone was able to suppress IFN-γ–stimulated STAT1 activity in monocytes, but not in T cells, and many of the suppressive effects of dexamethasone required several days' preincubation to become apparent.[178] This lack of effect was confirmed in activated $CD8^+$ T cells,[179] and furthermore, the ability of dexamethasone-stimulated macrophages to phagocytose apoptotic cells is prevented by IFN-γ.[180] These data suggest that dexamethasone responsiveness might be attenuated in a T_H1 environment and might contribute to the glucocorticoid insensitivity seen in rheumatoid arthritis.[181]

Cigarette smoking

Interestingly, patients with asthma who smoke cigarettes also show resistance to the anti-inflammatory actions of corticosteroids, and this persists to some extent even in exsmokers.[182,183] Cigarette smoking is an oxidative stress and can affect several aspects of steroid function, including GR nuclear translocation[184] and effects on nuclear cofactors.[47,185] Importantly, these effects are reversed by antioxidants.[47,184,185] Intriguingly, there is a marked increase in oxidative stress in severe CR asthma.[186,187] Increases in markers of oxidative stress, such as 8-isoprostane, appear to be relatively resistant to treatment with steroids.[188] This suggests that anti-oxidants or NO synthase inhibitors, which would reduce the formation of peroxynitrite, might therefore be effective therapies in CR asthma.

As discussed above, cofactor recruitment to GR has been reported to be important in glucocorticoid functions. One of these important cofactors, HDAC2, has been reported to be reduced in activity and expression in bronchial biopsy specimens of elderly "healthy smokers."[189] In smokers with chronic obstructive pulmonary disease (COPD), there was an increased decrease in HDAC2 expression and activity, which correlated with disease severity within the lungs, airways, and BAL fluid macrophages.[189] Patients with COPD show little clinical responsiveness to glucocorticoid therapy, and cells from these subjects also have attenuated responses to exogenous glucocorticoids.[47] In addition, restoration of HDAC2 expression by means of transfection into BAL fluid macrophages from patients with COPD restores glucocorticoid function.[104] Therefore it is possible that reduced cofactor expression might attenuate glucocorticoid function in asthmatic subjects who smoke, and enhancing cofactor expression or activity might restore, at least in part, glucocorticoid function in these patients.

Finally, nuclear erythroid 2 p45-related factor 2 (Nrf2) is a redox-sensitive basic leucine zipper transcription factor that is involved in the transcriptional regulation of many antioxidant genes. Recent evidence from Rangasamy et al[190] suggests that in murine models of asthma, disruption of the Nrf2 gene leads to severe allergen-driven airway inflammation and hyperresponsiveness associated with pronounced mucus cell hyperplasia and infiltration of eosinophils into the lungs. This effect of Nrf2 disruption resulted in increased expression of IL-4 and IL-13. The data suggest that Nrf2-directed antioxidant pathways might act as a major determinant of susceptibility to severe allergen-mediated asthma.

Because familial CR is very rare and most CR asthma is an acquired condition resulting from abnormal inflammation or immune activation, other factors must play a role in glucocorticoid responsiveness.

Genetic predisposition

The environment and genetic variants account for approximately 50% of the risk of asthma.[191,192] Five asthma genes or gene complexes (ADAM33, PHF11, DPP10, GPRA, and SPINK5) have now been identified with the use of positional cloning,[192-194] and the functions of these genes in severe asthma are currently under investigation.[192] However, few studies have been conducted on large enough cohorts of patients to make meaningful conclusions about the differing roles of genetics and the environment in severe asthma. Despite the current technical limitations, because of the sheer scale of the data involved, it is likely that the future characterization of severe CR asthma will involve, at least in part, a genetic component.[191,192]

Interestingly, however, there is some information linking polymorphisms in IL-4 signaling with asthma severity

and glucocorticoid responsiveness. Thus a polymorphism in the IL-4 promoter linked to increased IL-4 gene transcription (C-589T) is associated with decreased lung function in a group of asthmatic subjects with symptoms of differing severity.[195] The frequency of the T allele is also greater in African American asthmatic patients (38%) than among white asthmatic patients who display a greater risk of presenting with CR asthma than white patients (12%)[126] and have a greater disposition to reduced glucocorticoid responsiveness.[196] Furthermore, this study reported that the T allele was significantly overrepresented in patients with CR asthma. The IL-4 receptor α sequence variant Q-576R has also been associated with asthma severity in a couple of studies.[197,198] In addition, analysis of combinations of polymorphisms showed that asthmatic patients carrying both the T allele of −33C>TIL4 and the A allele of 576Q>RIL4RA had an increased risk of severe persistent asthma.

Genetic analysis of patients with CR asthma in an Icelandic population might have demonstrated a gene expression fingerprint that predicts clinical glucocorticoid responsiveness. Messenger RNA–isolated PBMCs obtained from 64 patients with CS asthma and 42 patients with CR asthma were examined by using a microarray under resting conditions or after stimulation with TNF-α or IL-1β. The expression pattern of 15 genes was able to distinguish dexamethasone responders (n = 26) from nonresponders (n = 18). Interestingly, one gene, p50 NF-κB, was able to predict glucocorticoid responsiveness in 81% in an independent cohort of 79 patients, although links with other interesting genes, such as the IL-4 receptor, STAT4, and MKP-2, were also reported. The utility of this work in a wider context has yet to be determined[199] because it is clear that genetic factors associated with severe asthma, glucocorticoid responsiveness, or both might be related to racial groups.[200]

Viral infection

Recurrent exacerbations are a major cause of morbidity and medical expenditure in patients with asthma. In a recent study patients with more than 3 severe exacerbations (n = 39) in the previous year were compared with those with only 1 exacerbation per year (n = 24).[119] Recurrent respiratory tract infections were included among factors significantly associated with frequent exacerbations (odds ratio, 6.9). Of interest is the fact that respiratory viruses are important exacerbation triggers,[201] and recent evidence has suggested that rhinoviral infection can reduce GR nuclear translocation and reduced corticosteroid function.[202] Overall, recurrent exacerbations that are associated with severe asthma are associated with specific comorbid factors that are easy to detect and that are treatable. Therapeutic interventions aimed at correcting these factors are likely to reduce morbidity and medical expenditure in these patients.

Allergen exposure

Patients with severe allergic asthma often get worse during the pollen season and require increased amounts of glucocorticoids to control their disease.[118] In addition, patients who are allergic to pet allergens who live with these animals require greater glucocorticoid treatment,[118] and similar effects are seen with other environmental allergens.[203] The Denver group has examined the effect of allergen exposure on GR function and GR-binding affinity in PBMCs from atopic asthmatic patients.[204] During the ragweed season, GR ligand-binding affinity was reduced in PBMCs, and this effect could be mimicked *in vitro* by exposure of cells to cat allergen for 48 hours. These effects were allergen specific because *Candida albicans* had no effect on GR-binding affinity in patients who were not allergic to this allergen. These effects on GR ligand binding correlated with reductions in attenuation of T-cell proliferation and could be reversed by antibodies to IL-2 and IL-4.[204] Importantly, asthma morbidity, mortality, and health services use are highest among inner-city populations, who have the least evaluations for allergen sensitization, little allergen avoidance education, and the least patient adherence with the National Asthma Education and Prevention Program Expert Panel recommendations.[203]

Microbial superantigens

The T-cell repertoire of patients with poorly controlled asthma has been examined.[205] TCR-BV8$^+$, but not other TCR-BV$^+$, T cells were significantly increased in these patients in both CD8$^+$ and CD4$^+$ cells, suggesting activation by a microbial superantigen.[206] In subsequent studies dexamethasone was shown to have a reduced ability to suppress T-cell proliferation in cells stimulated with a prototypic superantigen, staphylococcal enterotoxin B, compared with cells stimulated with PHA.[207] This study also suggested that the mechanism for this effect was mediated through the induction of GRβ.[207] More recently, superantigen-induced suppression of glucocorticoid responsiveness has been linked to ERK MAPK.[208] Superantigen preferentially activates the ERK pathway, leading to phosphorylation of GRα and reduced nuclear translocation. This effect was reversed by pharmacologic inhibition of the ERK pathway.[208] These observations suggest that bacterial or viral agents secreting superantigens might contribute to poorly controlled asthma and reduced glucocorticoid sensitivity.

Neutrophilia

Wenzel et al[209] investigated the cell profile of BAL fluid from patients with severe CR asthma taking high-dose oral glucocorticoids compared with that of patients with CS asthma and healthy subjects. Eosinophil counts were lowest in the patients with severe asthma receiving glucocorticoid treatment, being similar in level to those seen in healthy subjects. In contrast, eosinophil levels were highest in patients with moderate asthma not receiving glucocorticoid therapy. In comparison, neutrophil levels were significantly higher in the severe asthma group compared with those in the other 2 groups, suggesting a distinct form of inflammation in patients with severe asthma despite treatment with high-dose oral corticosteroids.

Importantly, despite neutrophils being described as glucocorticoid insensitive, it is becoming clear that various aspects of neutrophil function, including prevention of apoptosis, are controlled by glucocorticoids.[210-212] In respect to CR asthma, it is interesting to note that neutrophils express high levels of GRβ mRNA and protein at baseline and after IL-8 stimulation.[153] This might be a result of a specific action of serine-arginine–rich protein p30 because antisense oligonucleotides to this protein prevent the formation of the GRβ splice variant.[213]

MANAGEMENT OF GLUCOCORTICOID RESISTANCE

The management of patients with CR asthma poses a considerable challenge to the clinician. These patients are often subjected to the unwanted side effects of prolonged systemic glucocorticoid therapy in situations in which there is no evidence that it is exerting any appreciable benefit. A previous review in the *Journal*[118] clearly shows in detail the stepwise algorithm for treating patients with CR asthma, and this is summarized here. As described earlier, similar percentages of patients referred to The Brompton Hospital and to Denver for severe asthma have CR asthma.[123,126,127]

Step 1. Obtain a thorough history, physical examination, and appropriate laboratory tests to confirm the diagnosis of asthma. In the case of patients presenting with glucocorticoid-resistant asthma, it is also critical to rule out concomitant medical disorders, such as vocal cord dysfunction, gastroesophageal reflux, tracheomalacia, and chronic sinusitis.[6]

Step 2. Identify and remove potential allergens that might trigger the patient's disease and institute appropriate environmental controls at home, in school, and at work, with the focus on areas where the patient spends the greatest time.[203]

Step 3. Review the patient's inhaler technique. This should be incorporated as routine during the physical examination.

Step 4. Rule out psychosocial factors affecting the illness and compliance with treatment regimens.[123,214] Develop strategies to increase adherence to therapy, including simplifying the medication regimen and implementing an action plan.

Step 5. Evaluate asthmatic patients for potential microbial (eg, *Mycoplasma* and *Chlamydia* species) infection of the airways leading to other opportunistic infections. Such individuals might respond to a long-term course of clarithromycin.

Step 6. Maximize combination therapy for control of disease symptoms. Combination therapy improves symptom control and adherence.[215,216]

Step 7. Evaluate systemic corticosteroid pharmacokinetics to maximize pulmonary function with oral corticosteroids.[118] These are also useful to measure compliance, and intramuscular triamcinolone should be considered as an alternative.[119,125] Patients with poor absorption of prednisone frequently respond well to oral liquid steroid preparations. In patients with rapid corticosteroid elimination, a split-dosing regimen, with the second dose of the day administered in the afternoon, should be considered. In such patients the morning dose should be titrated, followed by conversion of the afternoon dose to the morning dose and an attempt to reduce to alternate-day therapy.

Step 8. Assess evidence for persistent tissue inflammation despite treatment with high-dose glucocorticoids (eg, exhaled NO, sputum eosinophils, or BAL-biopsy specimens).[118,217,218] This is most useful before and after a 1- to 2-week course of oral prednisone therapy.

Step 9. Consider alternative anti-inflammatory and immunomodulatory approaches (eg, anti-IgE, immunotherapy, cyclosporine, or methotrexate).[118,219,220]

In the future, more information is also needed on the pathology of severe asthma to determine whether there are ultrastructural abnormalities present that might be irreversible and might be glucocorticoid resistant.[209] It is important to determine whether glucocorticoid therapy can affect inflammation, components of airway remodeling, or both or whether individual patients have a noninflammatory drive to their disease.[118] In these latter patients, glucocorticoid treatment will not be effective and will lead to adverse side effects, and maximal bronchodilator therapy should be used for these patients.

There have been no systematic studies examining the long-term prognosis of patients with CR asthma. The major concern with this group of patients is that they might be at high risk for morbidity and mortality caused by asthma and the adverse effects of therapy, especially high-dose and long-term steroid therapy, that might alter their quality of life.[123,221] Furthermore, patients taking high-dose glucocorticoids must be monitored carefully for adverse effects related to glucocorticoid therapy, such as osteoporosis monitored by bone density, and measures should be initiated to minimize their effect, such as providing adequate dietary calcium and vitamin D.[35,222,223]

Therapeutic implications

Recently, the introduction of hydrofluoroalkanes (HFAs) as propellants in pressurized metered-dose inhalers (pMDIs) has allowed for the production of drugs with smaller particle size, leading to a 4- to 5-fold increase in lung deposition and increased delivery to the small airways.[224] Treatment with inhaled glucocorticoid administered with HFA-based pMDIs allows similar control of asthma symptoms with lower doses of the same drug as when administered through non-HFA pMDIs devices.[224] It is tempting to speculate that the long-term treatment of CR asthma with these new HFA-based pMDIs could potentially improve inhaled corticosteroid (ICS) efficacy. In addition, the safer side-effect profile of new glucocorticoids, such as ciclesonide, might enable higher topical doses to be administered.[225]

Furthermore, the multiple mechanisms underlying glucocorticoid insensitivity raises the possibility that this might indeed reflect different diseases with a common phenotype, and studies examining the efficacy of potential

new agents should be targeted toward subgroups of patients with severe CR asthma who clearly require effective new drugs and other approaches to improved asthma control.[226] For example, a recent study of an anti-$CD4^+$ T-cell antibody showed beneficial effects in a group of patients with CD asthma.[227] In addition, treatment with anti-IgE therapy in a small cohort of these patients with CR asthma has also shown clinical effectiveness.[219]

The overexpression of IL-2 and IL-4 in patients with CR asthma and their effects on glucocorticoid function suggest that antibodies or soluble receptors directed against IL-4 or IL-2 might prove effective. Indeed, in a small open study in patients with CR asthma with inflammatory bowel disease, anti-IL-2 therapy appeared very beneficial.[228] MAPK activity might be increased in key cells in patients with CR asthma, and this might affect glucocorticoid function.[226] Many agents targeting these pathways are in clinical development, and it might be possible to prevent AP-1 activation, for example, by using JNK inhibitors alone or, more likely, in conjunction with glucocorticoids to reduce inflammation in CR asthma and restore glucocorticoid sensitivity.[229] In a similar manner, the development of p38 MAPK inhibitors might prove a useful add-on therapy in CR asthma.[230] In addition, enhanced AP-1 activity might be due to the marked increase in oxidative stress seen in severe asthma,[217] suggesting that a potent antioxidant therapy might prove effective either alone or, more likely, as a restorer of steroid sensitivity. Inhibitors of NO synthase 2, by preventing peroxynitrite formation, might perform a similar role.

Many of the anti-inflammatory effects of glucocorticoids appear to be mediated through inhibition of NF-κB, and genetic studies implicate the NF-κB pathway in CR asthma.[199] Small-molecule inhibitors of IKK-2 are in development,[230] and recent evidence suggests that IKK2 inhibitor can prevent activation of inflammation induced by IFN-γ,[231] an event that is in part glucocorticoid insensitive.[177] There are worries about possible side effects of these drugs, such as increased susceptibility to infections; however, as a corollary to this, if either glucocorticoids or aspirin were discovered today, they would be unlikely to be used in human subjects because of their low therapeutic ratio and their side effect profile. Inhibition of specific coactivators activated by NF-κB might prove to be useful targets, especially if they also repress the action of other proinflammatory transcription factors.[232] Alternatively, activation of corepressor molecules might have therapeutic potential.[233]

Some patients with CR asthma have airway neutrophilia, whereas others are associated with eosinophilia.[134,209] Hence is it possible that chemokine receptor antagonists, such as those directed against neutrophils (CXCR1/2) and eosinophils (CCR3), have the hope of preventing inflammatory cell recruitment to the airways of patients with CR asthma and perhaps as a consequence affect the resolution of airway remodeling.[135,226]

Other approaches that might prove useful in the future for CR asthma include the administration of anti-inflammatory cytokines, such as IL-10, or the induction of IL-10–secreting T regulatory cells by vitamin D3 in combination with a glucocorticoid, inhibitors of phosphodiesterase 4, JAK3, or anti-TNF-α and IL-1 therapies. Antileukotrienes (montelukast, 10 mg for 4 weeks) have been tested in CD asthma but did not prevent sputum eosinophilia, although this was in a small number of subjects.[234]

CONCLUSIONS

Enormous progress has been made in improving glucocorticoid treatment since the introduction of hydrocortisone as the first clinically used glucocorticoid in 1948. Extensive drug development has resulted in highly potent molecules, the pharmacokinetic profiles of which have been optimized to minimize systemic exposure and to target activity to the lung. The majority of asthmatic subjects of all disease severity respond well to these agents. Despite this, there are a number of subjects with asthma who do not respond to even high doses of inhaled or even oral glucocorticoids. Advances in delineating the fundamental mechanisms of glucocorticoid pharmacology, especially the concepts of transactivation and transrepression and cofactor recruitment, have resulted in better understanding of the molecular mechanisms whereby glucocorticoids suppress inflammation. It is clear that inflammatory mediators might produce CR asthma by means of multiple distinct mechanisms acting on a range of these mechanisms that are potentially both cell-type and gene specific. Understanding these events will lead to the rational design of drugs that target these aspects of GR function and potentially restore glucocorticoid sensitivity in patients with CR asthma.

Finally, it is also possible that some aspects of severe CR asthma pathogenesis, such as airway fibrosis or smooth muscle hyperplasia, can be regulated by glucocorticoids. It will be important to develop cheap, easy, and reliable biomarkers of inflammation and airway remodeling to monitor glucocorticoid responses and genetic markers of CR asthma so that such patients are started on alternative anti-inflammatory therapies as early as possible in the disease process.

The literature in this area is extensive, and many important studies were omitted because of constraints on space, for which we apologize. We would like to thank other members of the Cell and Molecular Biology Group for their helpful discussions.

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