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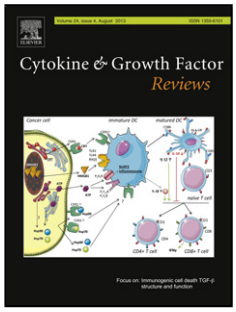

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Dominance of the strongest: inflammatory cytokines versus glucocorticoids

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ABSTRACT

Pro-inflammatory cytokines are involved in the pathogenesis of many inflammatory diseases, and the excessive expression of many of them is normally counteracted by glucocorticoids (GCs), which are steroids that bind to the glucocorticoid receptor (GR). Hence, GCs are potent inhibitors of inflammation, and they are widely used to treat inflammatory diseases, such as asthma, rheumatoid arthritis and inflammatory bowel disease. However, despite the success of GC therapy, many patients show some degree of GC unresponsiveness, called GC resistance (GCR). This is a serious problem because it limits the full therapeutic exploitation of the anti-inflammatory power of GCs. Patients with reduced GC responses often have higher cytokine levels, and there is a complex interplay between GCs and cytokines: GCs downregulate pro-inflammatory cytokines while cytokines limit GC action. Treatment of inflammatory diseases with GCs is successful when GCs dominate. But when cytokines overrule the anti-inflammatory actions of GCs, patients become GC insensitive. New insights into the molecular mechanisms of GR-mediated actions and GCR are needed for the design of more effective GC-based therapies.

INTRODUCTION

Synthetic glucocorticoids (GCs) are among the most potent and most effective anti-inflammatory drugs. GCs penetrate through the cell membrane and bind to the Glucocorticoid Receptor (GR), a transcription factor belonging to the family of nuclear receptors. Upon ligand binding, GR translocates to the nucleus, where the activated GR can either transactivate or transrepress specific genes. Transactivation of GR is predominantly mediated by binding of GR dimers to GC response elements (GRE) in the promoter region of GC-responsive genes, followed by recruitment of coregulators and induction of gene transcription. In addition, GR positively affects gene transcription by DNA-independent mechanisms. Next to transactivation, GR has also transrepressive capacities. The best known mechanisms of transrepression are the protein–protein interactions between monomeric GR and other transcription factors, such as NFκB and AP1. Thus, GR mediates either positive or negative transcriptional events, which together culminate in coordinated anti-inflammatory actions (1, 2).

Owing to their strong anti-inflammatory properties, GCs are often used for the treatment of many inflammatory and autoimmune diseases. However, despite the success of GC therapy, two major drawbacks are associated with the prolonged use of GCs. First, GC therapy is often accompanied by a wide range of detrimental side effects, such as osteoporosis and diabetes (3). In addition, subpopulations of patients display partial or complete lack of response to GCs, referred to as GC resistance (GCR) (4). The incidence of GCR is dependent on the inflammatory environment. Several inflammatory diseases, including sepsis, chronic obstructive pulmonary disease (COPD) and cystic fibrosis, seem to be largely steroid resistant, whereas only a minority of patients suffering from asthma, inflammatory bowel disease or rheumatoid arthritis respond poorly to the beneficial effects of GCs.

GCR is a serious clinical problem because it hampers the full therapeutic exploitation of the anti-inflammatory properties of GCs. Patients suffering from inflammatory diseases are often treated with alternative broad-spectrum anti-inflammatory therapies, although all of them are also likely to have serious side effects. An alternative treatment strategy is to reverse GCR by blocking the mechanisms causing it. Hence, detailed knowledge of the physiological actions of GCs and of the negative effects on these is crucial. We review the current knowledge of the mechanisms that participate in GR signaling and how they contribute to GC sensitivity or

insensitivity. As inflammatory cytokines, such as TNF, and their respective signaling pathways play crucial roles in the pathology of most immune diseases and as these may inhibit GR function (5, 6), we will discuss these mechanisms of GCR in detail. We believe that understanding the effects of inflammatory cytokines on GR signaling and the mechanisms of these effects may reveal novel therapeutic targets for reversal of GCR.

MOLECULAR MECHANISMS OF GC ACTION

GC biosynthesis and metabolism

GCs are a class of stress-induced steroid hormones synthesized by the adrenal cortex. Their production is tightly regulated by the hypothalamic–pituitary–adrenal (HPA) axis, more specifically, by the production of CRH in the hypothalamus and ACTH in the anterior pituitary. GCs are critical for normal physiological function due to their regulatory effects on carbohydrate, lipid and protein metabolism, development, and cell differentiation. In basal conditions, endogenous GCs are secreted in a classic circadian and ultradian pattern, i.e. recurrent periods or cycles repeated throughout a 24 hour circadian day, with peak secretion in the morning. GCs are also released in response to diverse stimuli, including stress and inflammatory cytokines, and are therefore also important regulators of the immune response.

In the circulation, most of the secreted GCs are bound to corticosteroid-binding globulin (CBG) and to a lesser extent to albumin. The circulating level of GCs is additionally regulated through its negative feedback activity on both CRH and ACTH release (7). Due to their lipophilicity, free GCs can readily diffuse across cellular membranes to exert their effects. Intracellularly, the bioavailability of GCs is regulated by the 11-β hydroxysteroid dehydrogenase system (11-βHSD). These enzymes catalyze the conversion of inactive cortisone to its biologically active form, cortisol, and vice versa.

GR activation

Unliganded GR is sequestered in the cytoplasm within a chaperone complex that includes heat shock proteins HSP90 and HSP70, and the HSP90-binding molecules p23 and FKBP51. Upon binding of GCs, GR undergoes a conformational change that drives its translocation into the nucleus. The chaperone complex is partly dissociated but is still required for nuclear transport of GR. The mechanism of nuclear import involves a switch in binding of FKBP51 to FKBP52

and necessitates the nuclear proteins importinα and importin13. Although inactive GR is mainly found in the cytoplasm, GR is not rigidly compartmentalized but constantly shuttles between nucleus and cytoplasm. Nevertheless, GR performs most of its activities in the nucleus (8).

The chaperone complex contains a phosphatase, which assists in the dephosphorylation of newly formed GR. Upon ligand binding, GR is phosphorylated by different kinases, including CDK and MAPK. GR protein contains several phosphorylation sites, and the phosphorylation pattern modulates its transcriptional activity (9). GR is subject to various other posttranslational modifications that also affect its receptor function and thereby serve as an additional important mechanism to regulate the cell-specific responses to GCs. Protein phosphorylation facilitates GR ubiquitination and sumoylation. GR is ubiquitinated on the conserved lysine residue 426 located in a PEST degradation sequence (10), and consequently GR is degraded through the ubiquitin-proteasome pathway (11). Three consensus sumoylation sites have also been identified in GR, and they either potentiate or repress its activity (12). For example, upon phosphorylation of Ser226 by JNK, GR is targeted for sumoylation, reducing its stability (13). Moreover, nitrosylation and oxidation of GR can negatively affect its function by decreasing GR ligand binding (14) and by disturbing GR nuclear translocation and DNA binding (15), respectively. Furthermore, acetylation of GR, for example by the circadian rhythm-generating transcription factors CLOCK and BMAL1, can also lead to reduced transcriptional activity (16).

GR actions

The ligand-induced conformational changes and posttranslational modifications of the GR protein determine the selective recruitment of coactivators and corepressors. By modifying and remodeling the chromatin structures, these cofactors ensure highly coordinated regulation of gene transcription (17, 18). Some coactivators, including SRC2 (steroid receptor coactivator) and CBP/p300, alter the acetylation status of histones, whereas the SWI/SNF complex has chromatin remodeling activity (19). Together, these activities lead to nucleosome repositioning and chromatin relaxation, followed by recruitment of mediator proteins and the TATA-binding protein (TBP), enabling transcription by RNA polymerase II of several metabolic and anti-inflammatory genes by GR (20). Hence, cofactors play a crucial role in the responsivenesss to GCs.

Positive regulation of gene expression is usually attributed to binding of GR to conserved glucocorticoid response elements (GREs). The consensus GRE sequence is the palindromic motif 5'-AGAACAnnnTGTTCT-3'. Activation of gene transcription *via* GREs depends on direct binding of GR homodimers. Interestingly, the use of ChIP-seq technologies has revealed that many GR binding sites are located far (> 5000 bp) from the promoter proximal region of target genes and that they lack a consensus GRE element (18, 21). This can be explained by the presence of regions containing composite GR binding sites that require the binding of a monomeric GR together with another transcription factor. In addition, monomeric GR can positively interfere with the transactivation capacity of other transcription factors by a tethering mechanism, thus affecting gene transcription without binding to DNA.

Moreover, tethering of pro-inflammatory transcription factors by GR, such as AP1, NFκB, IRF3 and STAT5, can also repress inflammatory gene induction and thereby contribute to the anti-inflammatory actions of GR (22). This tethering mechanism involves multimeric complexes of different proteins. The recruitment of corepressors, such as HDAC2, TRIP6 and STAMP, is required for the binding of GR to transcription factors. For example, deacetylation of GR by HDAC2 is involved in the interaction of GR with NFκB (23). Recently, however, it was reported that HDACs can also play a positive role in GR transactivation (24). Similarly, GRIP1, a well-known coactivator of GR, is also used as a corepressor by GR to suppress NFκB, AP1 and STAT1 activity (25), further evidenced by a recent ChIP-seq study showing binding of GRIP1 to GR- and p65-bound promoters (21). Moreover, GR negatively regulates gene transcription by binding as a monomer to nGRE sequences (negative glucocorticoid receptor response element) (26, 27). These palindromic sequences separated by spacers of less than three nucleotides have been identified in more than 1000 genes. Repression of these genes by GR also involves the recruitment of corepressor complexes, including NCoR and SMRT (26). However, few inflammatory genes have been reported to be regulated by GR *via* nGREs. Furthermore, there exist reports on gene repression by competition for an overlapping binding site of another transcription factor, sequestration of a DNA-bound transcription factor and competition for common cofactors. Altogether, this complex GR-mediated transcriptional control ensures the strong anti-inflammatory potential of GCs.

GR interaction with DNA and changes in chromatin structure are extremely dynamic, with a turnover of a few seconds, which allows appropriate and fast cell-type specific responses to GCs (28). In general, GR actions are highly dependent on cell-type, as illustrated by the

finding that different cell types display a low degree of overlap in GR binding sites (21, 29, 30). These datasets also clearly indicate that GR binds almost exclusively at pre-accessible chromatin sites (31) by prior binding of other transcription factors, such as AP1 and STAT3, which recruit chromatin remodelers (32, 33).

A detailed understanding of the molecular mechanisms of GC action is needed to understand GCR. The inflammatory environment can negatively alter GC actions in many different ways, many of which will be discussed in next chapter.

INFLAMMATION-MEDIATED MECHANISMS OF REDUCED GC ACTIONS

Reduced responses to the beneficial anti-inflammatory effects of GCs may result from defects at different levels of the physiological actions of GCs/GR, including reduced GC binding to GR, lower GR expression, impaired nuclear translocation, reduced ability of GR to bind DNA, and altered cofactor activity. These events can be modulated by inflammatory cytokines and their signaling pathways, which may lead to a weak clinical response to GC therapy of inflammatory diseases. We review here the effects of cytokines on the different levels of GC physiological actions.

Altered ligand availability

Acquired GCR may relate to alterations in the bioavailability of GCs, which can be influenced by inflammatory cytokines. Several pro-inflammatory cytokines are known to activate the HPA axis, resulting in enhanced secretion of GCs (34). Other events preceeding receptor binding include effects on corticosteroid-binding globulin (CBG) or on steroidogenic enzymes. Cytokines can inhibit the hepatic secretion and synthesis of CBG and increase its catabolism at inflammatory sites (35, 36). Following TNF stimulation of rats, a strong decrease in CBG levels was observed (37). These mechanisms increase the availability of circulatory free GCs. In addition, inflammatory cytokines increase 11βHSD1 expression and enzyme activity in different cell types (38-40), for example by TNF in lung A549 epithelial cells (41). Thus, cytokines seem to amplify GC bioavailability. However, chronic exposure to increased GC levels can lead to GCR due to homologous downregulation of GR expression (42).

On the other hand, increased levels of 11βHSD2 reduce the amounts of intracellular bioactive GCs. Recent reports have shown that the inflammatory kinases p38 and ERK1/2 can reduce the transcription of 11βHSD2 in trophoblast cells (43, 44). Decreased intracellular GC levels can also result from the increased expression of the membrane cassette transporter P-glycoprotein, which mediates the efflux of GCs out of cells, a process that is involved in the development of GCR (45). Interestingly, a causative role for TNF in the latter mechanism has been postulated (46).

Reduced GC binding to GR

In peripheral blood mononuclear cells, IL2 and IL4 reduce the affinity of GCs for GR (47). This observation indicates that pro-inflammatory cytokines, by reducing GC-GR binding, may be involved in the development of GCR in certain inflammatory diseases in which these cytokines play prominent roles, such as asthma. GR contains several cysteine residues that are critical for ligand binding. Nitrosylation of GR also results in defective ligand binding (48). The relation between high levels of nitric oxide and GC insensitivity, due to inhibition of GR ligand binding, has been observed in endotoxemic rats (49). In contrast, the addition of an NO group to prednisolone, a synthetic GR ligand, enhances GC function following donation of the NO moiety to GR (50). Other studies also show enhancement of GR activity by NO (51). These conflicting results might be explained by differences in the nitration targets. Alternatively, the effects on GR function might depend on the cell type or on the NO concentration used. Although a direct effect of cytokines on nitrosylation of GR has not been reported, it is well known that cytokines can induce NO synthase expression. In inflammatory diseases, expression of inducible NO is often increased, as has been observed in sepsis patients (14). Therefore, the inflammatory environment might influence GC sensitivity by altering NO levels.

Reduced GR levels

Nitrosylation can also affect GR expression levels. One study found that neuronal NO synthase can decrease GR expression (52), whereas another one found that GR expression is upregulated by NO (53). The effect of cytokines on GR expression is also controversial, and both upregulation and downregulation have been reported. This discrepancy is probably due to differences in experimental conditions and assay strategies. IL1α and TNF have been reported to downregulate GR in a rat hepatoma cell line and in monocytes, respectively (5,

54). These effects are cytokine- and cell-type specific, as in mouse fibroblasts, IL1α also inhibits GR function, but by blocking GR translocation (55), and TNF does not reduce GR levels in hepatoma cells (our unpublished results). In contrast, TNF does reduce the *in vivo* hepatic levels of GR, partly contributing to the reduced transcriptional actions of GR in mice injected with TNF (56). It is clear that the cellular GR levels are dynamic and can be influenced by cytokines. This implicates another means by which the inflammatory environment can affect GC responses as the GR levels are a major factor determining the sensitivity to GCs and are critical for regulating the transcriptional outcome (57) .

The concentration of available ligand is also an important determinant of GR levels, as GCs downregulate both GR mRNA and protein levels by a process called homologous downregulation. Note that T cells are an exception to this rule: GCs auto-upregulate GR in these cells (58). GC-induced reduction of GR mRNA levels is attributed to inhibition of transcription initiation by the presence of an nGRE in exon 6 (59). As mentioned above, inflammatory cytokines increase intracellular GC levels, and the observed effects of cytokines on GR expression *in vivo* might be indirectly mediated by GCs. In addition, several mechanisms have been proposed for the regulation of GR mRNA stability. The AU rich elements (AREs) in the 3'UTR of GR mRNA might mediate GR destabilization of GR by binding RNA destabilizing proteins, such as tristetraprolin (TTP) (60). Cytokines, such as TNF, induce TTP levels and activate TTP activity via MAPK activation (our unpublished findings) and in this way they might destabilize GR mRNA.

Studies have also focused on the effects of inflammatory cytokines on the expression of GR isoforms. Alternative splicing of exon 9 produces two distinct mRNA molecules that generate two GR variants, the conventional, ligand binding receptor (GRα) and another isoform, GRβ (61). GRβ can bind to GRE binding sites and can also form heterodimers with GRα (62). By preventing GRα from binding to GREs, GRβ may act as a dominant negative inhibitor of GRα-mediated actions (61). The ability of GRβ to modulate transcription, independent of GRα, might also contribute to the antagonistic effects of GRβ on GRα actions (63). Therefore, GRβ is likely involved in the regulation of tissue-specific sensitivity to GCs (64). Exposure of various inflammatory cell types to cytokines increases the expression of GRβ (6). For example, IL17 and IL23 induce GCR in PBMCs *via* GRβ upregulation (64). So, an imbalance in the relative levels of GRα and GRβ could underlie the GC insensitivity associated with clinical conditions in which these cytokines play an important role, although the relative

levels of GRβ remain much lower than those of GRα in inflammatory cells. Certain epithelial cell types, however, show high expression levels of GRβ, so this mechanism of GCR may be of particular relevance in epithelia (65). It has also been shown that TNF and IL1 can, through NFκB binding sites located in the GR promoter, double the GRβ expression level while increasing GRα by only 1.5-fold, (6). The promoter region of the GR gene, *NR3C1*, contains numerous binding sites for several inflammatory transcription factors, such as AP1 (66) and IRF1 (67). These transcription factors are known to upregulate GR expression, whereas GRF1 and c-EST1/2 can repress it (58). Thus, inflammatory environments in which these transcription factors are predominantly active can influence GR expression at the level of transcription. Moreover, other mechanisms are involved in the turnover of the GR protein, including proteasome mediated degradation, which is mediated by ubiquitination of lysine 419 in the PEST sequence (10, 11). The roles of cytokines and their signaling pathways in this process have not been elucidated yet.

Another level of fine-tuning GR levels is provided by microRNAs (miRs), which are small non-protein encoding RNAs acting as posttranscriptional regulators with prominent roles in inflammation. Several miRs, including the ubiquitously expressed miR18 and the brain-specific miR124a, have been shown to decrease GR protein levels (68, 69). MiR124a is upregulated by GCs (70) and might be involved in the GC-mediated homologous downregulation of GR. In addition, more recent reports have shown that GR transcripts are targeted by many more microRNAs, such as miR-96, miR-101a, miR-142-3p, and miR-433, all of which downregulate GR expression levels by up to 40%. As these miRs are upregulated by ACTH and inflammatory cytokines can induce ACTH, inflammatory mediators might limit the GC response by GR downregulation indirectly *via* the induction of miRs (69). The interplay between inflammatory cytokines, miRs and GR functions will likely become an important field of investigation, especially in relation to GCR.

Impaired GR nuclear translocation

The integrity of the mature GR heterocomplex is required for optimal ligand binding. As discussed above, GR binding of GCs results in a conformational change in the chaperone complex to enable its translocation to the nucleus. Therefore, alterations in the GR chaperone profile contribute to changes in GC responsiveness (65). For example, the relative levels of FKBP51/52 are associated with the intensity of the GC response, more specifically, the more

FKBP51, the lower the GR ligand affinity, the less nuclear translocation and the less active GR (71, 72). The role of HSP70 and HSP90 chaperone proteins is, however, controversial. Alterations in HSP90 and HSP70 were associated with decreased cellular sensitivity in several studies (73, 74). On the contrary, GCR in pediatric acute lymphoblastic leukemia patients was not correlated with the expression levels of HSP90/70 (75). Although there are no reports on a direct effect of cytokines on chaperone levels or on recruitment in the GR complex, a regulatory role for IL2 on HSP90 activity has been documented (76). In addition, the cofactor HDAC6 can regulate GR function indirectly by reducing acetylation of HSP90 and thereby nuclear translocation of GR (77). Furthermore, oxidative stress can lead to disruption in the chaperone complex resulting in impaired nuclear translocation (15).

Nevertheless, several cytokines have been shown to impair GR nuclear translocation. For example, it was shown that IL1β in combination with H_2O_2 impairs importin7-mediated nuclear transport of GR, leading to GC insensitivity (78). In addition, IL2 and IL4 reduce GR translocation in T cells (79), whereas IL13 has this effect in monocytes (80) and IL1α in fibroblasts (81, 82). The pro-inflammatory MAPK p38 might be important in mediating these cytokine-induced negative effects on GR translocation by phosphorylating GR, as the effect of IL2 and IL4 is blocked by a p38 inhibitor (83).

Altered GR posttranslational modifications

As phosphorylation modulates cellular trafficking, posttranslational modification must also be an important determinant of GC sensitivity. GR is a phosphoprotein containing several phosphorylation sites, including Ser113, Ser141, Ser203, Ser211, Ser226 and Ser404 in the N-terminal domain of GR. Changes in its phosphorylation pattern may affect all aspects of its function. For example, in lung cells the degree of Ser211 phosphorylation correlates with ligand binding, nuclear translocation, and eventually GR transcriptional actions. TNF and IFNγ reduce Ser211 phosphorylation in airway smooth muscle cells by increasing the levels of the PP5 phosphatase and in this way reduce GR transcriptional activity (84).

Various kinases, such as MAPK, can phosphorylate GR, although with different functional outcomes. Many pro-inflammatory cytokines induce p38, JNK and ERK, all of which have been shown to inhibit GR function. For example, JNK phosphorylation of GR at Ser226 results in enhanced nuclear export and proteasomal degradation (85) and IL2 and IL4 reduce GR activity by p38 MAPK induced phosphorylation of GR (83). However, phosphorylation

of GR by p38 MAPK remains controversial. The activation of p38 in HeLa cells also impairs GR activation by impairing ligand binding (86), whereas in lymphoid cells p38 increases phosphorylation on Ser211, resulting in increased transcriptional activity of GR (87). These findings underscore the stimulus-, cell- and species-specific nature of GC insensitivity. In addition, a recent study showed that not all GC–activated genes are similarly regulated by p38. GR activity is promoter-specific dependent on the transcription factor bound to the promoter region and the GRE sequence itself (88). Moreover, p38 retains GR in an inactive state in the absence of ligand in airway smooth muscle cells (89), which obviously also affects the overall GC responsiveness. It is becoming clear that GR can be phosphorylated on many other serine, threonine and tyrosine residues, which indicates that other inflammatory signaling pathways could converge on GR to alter GC signaling. Interestingly, reduced transcriptional activity of GR results in reduction of MKP1 expression. As MKP1 normally dephosphorylates and inactivates MAPK, reduced MKP1 levels will likely result in increased phosphorylation of GR, thus implying a potential enhancing negative effect on GR activity.

Altered phosphorylation of GR can also change its nuclear/cytoplasmic shuttling by targeting GR for ubiquitin-mediated proteasomal degradation, which affects the receptor half-life (11). In addition, JNK-dependent GR phosphorylation increases the sumoylation status of GR and thereby decreases the transcriptional activity of GR (13). In contrast, it was recently reported that sumoylation on lysine 703 of GR potentiates its activity (12). This shows that GR is subjected to various posttranslational modifications, all of which affect GR function in different ways. The acetylation status of GR also determines the transcriptional outcome, as it is required for transcriptional induction by GR, whereas its deacetylation by HDAC2 allows it to associate with and repress NFκB (23). Consequently, inhibition of HDAC2 by nitrosylation leads to GC insensitivity in COPD patients (90). However, not much is known about the influence of inflammatory cytokines on the activity and ratio of HAT/HDACs. Similarly, although cytokines such as TNF are well known inducers of oxidative stress (91), an effect of cytokines on GR function *via* oxidation has not been described. Nevertheless, GR is a redox-dependent transcription factor and its oxidation reduces its DNA binding capacity (92, 93). Oxidative stress can also indirectly influence GR function by decreasing the expression and activity of HDAC2 (94, 95).

Negative effects on GR function by transcription factor activation

Pro-inflammatory cytokines exert their actions by activating transcription factors. In turn, pro-inflammatory transcription factors can affect GR function in several ways. For example, ERK is involved in the TNF-induced suppression of GR translocation in HACAT cells (96). It has already been reported that GR nuclear translocation is abrogated by a cotreatment of IL2 and IL4 *via* p38-mediated phosphorylation of GR (83), whereas in T cells IL2-mediated reduction of GR nuclear translocation involves STAT5 (97, 98). The latter is mediated by direct binding of STAT5 with GR, a mechanism shared with many other transcription factors, including NFκB, Smad6, T-bet and AP1 (97, 99). Later, it was shown that the interaction of GR with AP1 or NFκB is mediated by nTrip6 (100). The SWI/SNF complex could also be involved in the crosstalk between GR and NFκB (101). The crosstalk mechanism, at least for NFκB and AP1, is based on a mutual antagonistic interaction (29). NFκB, AP1 and other transcription factors can be activated by pro-inflammatory cytokines, such as TNFα, and play pivotal roles in mediating inflammatory and immune responses. In this way, the increased inflammation observed in inflammatory disorders can result in reduced GC responsiveness. Note that tethering of STAT3 to GR results in synergism rather than transcriptional repression (99). Recent papers have shown that the latter principle is also applicable to other main pro-inflammatory transcription factors such as NFκB (41).

The binding of GR to its binding sites is mainly determined by the cell-type specific chromatin landscape (31), and in most cases by the presence of already-bound pioneering transcription factors, such as AP1, CREB1 and FOXA1, which help to open the chromatin (102-104). As GR is predominantly recruited to pre-accessible chromatin sites (105), these transcription factors play a crucial role in GR transcriptional regulation (32). For instance, C/EBP primes the chromatin for GR recruitment (52). It has been postulated that the loss of C/EBP in airway smooth muscle cells of asthmatics leads to loss of GR function (106). Accordingly, GR regulates gene expression through direct and/or indirect interaction with numerous transcription factors, such as AP1, Oct, C/EBP, NFκB, and STATs (29, 99, 103, 107). Histones can also be modified posttranslationally and can determine the chromatin structure and recruitment of transcription factors (108). However, whether this is influenced by the inflammatory environment needs to be addressed.

Abnormal chromatin environment

The GR transcriptional outcome is determined by the chromatin structure and by factors that regulate chromatin accessibility (31, 105), which are dependent on the inflammatory environment. Coactivators alter the chromatin structure, making it more accessible for the assembly of general transcription factors and the RNA polymerase complex, resulting in increased transcriptional activity by GR. Among the general coactivators are CBP/p300 and members of the p160 cofactor family, which consists of SRC1, SRC2/GRIP2 and SRC3. Owing to the important role of coactivators, changes in cofactor recruitment are implicated in impairment of the transcriptional effects of GR. Inflammatory transcription factors such as NFκB and STATs compete with GR for several general coactivators (17) and in this way determine the efficiency of GR transcriptional actions. For example, overexpression of CBP, SRC1 and SRC3 attenuates the NFκB mediated repression of GR activity (109). In addition, IRF1, induced by TNF and IFNγ, can reduce GR actions by competing for binding to GRIP1 coactivator, leading to GC insensitivity in airway smooth muscle cells (110, 111). As most of these studies used transient overexpression experiments, the *in vivo* role of cofactor competition remains to be addressed. Interestingly, GRIP1 is involved in both transactivation and transrepression actions of GR. However, decreased levels GRIP1 show only reduced transrepression activity of GR but no effect on transactivation (21).

Furthermore, the expression levels and activity of the interacting coregulators also influence the sensitivity to GCs. In particular, TNF can suppress the expression of SRC1 and SRC2 in smooth muscle cells (112). Cofactors are also subject to several posttranslational modifications affecting their activity, localization and half-life, and consequently their GR binding (113). In this way, kinases may indirectly affect GR responsiveness by modulating GR cofactors. Recently, GC-dependent phosphorylation of GRIP1 was shown to be important for GR–GRIP1 binding (114). Although it is reported that pro-inflammatory cytokines can inhibit GR function indirectly by ERK phosphorylation of a GR cofactor (17), the general effect of inflammatory mediators on cofactor post-transcriptional changes remains not well known. Note that the cofactor profile recruited by GR depends on the GR binding sequence (115). Recently, G9a was identified as a novel GR coactivator acting as a molecular scaffold for the assembly of other coactivators, but only for some GR target genes (116). Together this shows that, if inflammation alters the cofactor profile of GR, it will likely affect specific genes involved in certain tissues and diseases, rather than having more generic effects.

It is clear that reduced coactivator activity and other changes in GR physiology can negatively affect GR transcriptional induction. Thus, reduced expression of GR-inducible genes, of which many such as MKP1 and Gilz have prominent anti-inflammatory roles, will impair the anti-inflammatory potential of GCs (1). In addition, many of the anti-inflammatory actions of GR are mediated by blocking NFκB and other transcription factors, a process dependent on the binding of corepressors such as HDAC2 and Brahma-related gene 1. Inflammatory cytokines have been reported to reduce the activity of HDAC2, leading to GC insensitivity. To illustrate, IL17 reduces HDAC2 activity via p38/ERK in airway epithelial cells (117).

GC INSENSITIVITY IN INFLAMMATORY DISEASES

GCs are the most effective treatment for many inflammatory diseases. However, the occurrence of GC insensitivity in several inflammatory diseases suggests that inflammatory mediators modulate the cellular response to GCs. As mentioned above, inflammatory cytokines may promote the development of steroid insensitivity. As various cell types and cytokines are involved in the pathogenesis of inflammatory diseases, the mechanisms contributing to decreased GC sensitivity for a disease are heterogeneous. However, similar molecular mechanisms have been identified in different diseases, suggesting that the same therapeutic approaches could improve GC sensitivity. We will discuss various mechanisms that account for the reduced GC sensitivity associated with several inflammatory and autoimmune disorders, with a focus on asthma.

Asthma

In general, GC therapy is remarkably successful in the treatment of asthma, a heterogeneous disease characterized by airway hyperresponsiveness and inflammation. However, about 5–10% of patients are refractory to the anti-inflammatory effects of GCs (118). This sizeable subgroup is clinically defined by neutrophil-driven inflammation (119) and accounts for more than 50% of the total health care costs associated with asthma (118).

In vitro studies have used different lung cell types to examine the mechanisms of GC resistance in asthma. These showed that cytokines can induce GC insensitivity in both airway and immune cells, for example IL2 and IL4 in T cells (47, 98), IL13 in monocytes (80), IL27+IFNγ in macrophages (120), TNF+IFNγ and IL1β in airway smooth muscle cells (111,

121) and IL17 and TNF in airway epithelial cells (117, 122). Although reduced sensitivity to GCs has been clinically associated with neutrophilic airway inflammation (119), the *in vivo* cellular and molecular mechanisms are still largely unclear. Proposed mechanisms leading to steroid resistance in asthma include increases in inflammatory mediators and oxidative stress, both of which decrease the GR number or binding affinity (123). It is noteworthy that it is difficult to predict whether the reduced GC response is caused by the inflammatory setting itself, or else the inflammatory environment is less responsive to GCs, due to intrinsic defects in neutrophils for example.

Although several studies have reported negative regulatory effects of pro-inflammatory cytokines on GR, it is difficult to translate the findings from *in vitro* and mouse models to humans. This difficulty is partly due to the complex pathogenesis of asthma and the multiple cell types involved. IL17 leads to GC insensitivity in airway epithelial cells, increased IL17 levels have been observed in patients with severe GC refractory asthma, and a link between IL17 and neutrophilic inflammation was reported (124-126). Therefore, IL17 might play a role in the GC sensitivity in refractory asthma patients. Recently, it was proposed that vitamin D therapy, by inducing IL10 in regulatory T cells, can counteract the effects of IL17 on GR actions and hence restore GC sensitivity (127). However, this does not exclude a role for other cytokines, as steroid resistant asthma patients also have elevated levels of IL2 and IL4 (128). Also, IP-10 and GMCSF levels are significantly linked with neutrophilic asthma (129). However, whether these cytokines contribute to the development of GC refractoriness is not clear. Moreover, increased levels of TGFβ are detected in airway fluids of severe asthma patients and, as shown by *in vitro* studies, TGFβ can impair GC responses (130). Another important cytokine in immune disorders is TNF, which has also been implicated in asthma pathology. TNF levels are increased in patients with steroid refractory asthma (131), and TNF contributes to the development of a Th17-mediated neutrophil-rich environment (132). Therefore, anti-TNF based therapies have been tested, but the results are incongruous (133).

Furthermore, the mechanism by which these cytokines abrogate GR function might also be diverse. GRβ expression is increased in patients with GC insensitive asthma (62), and *in vitro* studies have shown that IL17, IL2 and IL4 increase GRβ expression (62, 64, 134). Therefore, it is likely that these cytokines reduce GC responses in asthma patients by inducing GRβ. However, in addition to the effect of IL17 on GRβ expression, it can reduce GC actions by activation of the PI3K pathway and consequent reduction of HDAC2 activity (117). Reduced

HDAC2 levels and activity have been extensively linked with GC insensitivity in asthma (135). Reduced HDAC2 expression and activity can also be caused by oxidative stress (*via* PI3Kó) and nitrative stress (4). In addition to the TR actions of GR mediated by HDAC2, a few GC resistant patients have defects in transcriptional induction by GR (136). This can be attributed to various defects in GC physiology, such as reduced GR expression, altered ligand affinity, reduced DNA binding, and reduced availability of coactivators, all of which can be induced by different cytokines.

Other inflammatory diseases

Many of the above mentioned inflammatory cytokines are also involved in the pathogenesis of several other autoimmune and inflammatory diseases in which GC insensitivity occurs. Therefore, although most GCR studies have focused on asthma, it is conceivable that similar mechanisms of GCR will be found in many other inflammatory diseases. For example, IL17 and TNF play a detrimental role in many inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis (131, 137). As these and other cytokines reportedly affect GC actions in different immune cells, they might also be involved in the onset of GC insensitivity in these diseases. Several links between TNF and the occurrence of steroid refractoriness have already been reported for inflammatory bowel disease (138, 139). Another interesting pro-inflammatory cytokine is MIF, which has been associated with numerous inflammatory diseases (140). MIF can block the anti-inflammatory actions of GR in patients with ulcerative colitis, rheumatoid arthritis or asthma (141-143).

On the other hand, multiple defects in GC/GR physiology contributing to reduction of GC responses have been identified in different diseases. For example, higher expression of 11βHSD type 2 was found in cells derived from patients with rheumatoid arthritis, which might result in glucocorticoid resistance (144). In addition, weaker expression of GR has been reported in patients with steroid resistant ulcerative colitis (145) and sepsis (70, 146). The downregulation of GR in sepsis patients has been linked with increased levels of miR124 (70). On the contrary, another study showed that expression and binding activity of GR is upregulated in septic patients (147). Next, a reduced HSP90:GR ratio was found in peripheral blood mononuclear cells from individuals with steroid resistant multiple sclerosis (74) and refractory asthma (148). Also, a role for GRβ was postulated in steroid resistant ulcerative colitis (149), sepsis (150) and rheumatoid arthritis patients (151). However, a possible

inflammatory cause for these defects remains to be elucidated. Nevertheless, GCR in Crohn's disease was shown to be linked with constitutive activation of epithelial NFκB, JNK and p38 by preventing GR transcriptional activity (152, 153).

THERAPEUTIC IMPLICATIONS

Given the effect of different pro-inflammatory cytokines and their signaling pathways on GR function (Figure 1), consideration should be given to the possibility of therapies directed at immunoregulatory cytokines. In this way, the use of additional treatments to restore the therapeutic effect of GCs represents a double-barrel approach to reducing inflammation: blocking the inflammatory pathway and enhancing the anti-inflammatory actions of GR. Interestingly, the use of inflammatory blockers, by avoiding GCR, may allow the use of lower doses of GR ligands. This might also reduce the side effects typically associated with long-term GC therapy. Current therapeutic strategies to restore the integrity of GR function involve targeting relevant inflammatory signaling molecules that appear to be deeply involved in GR disruption. Antagonists directed against IL2 receptors (CD25) were effective in GCR patients with inflammatory bowel disease (154). As similar mechanisms might lead to GCR in asthma patients, IL2R antibodies might also be effective in GCR asthma. In addition to direct cytokine blockers, the downstream signaling pathway might also be targeted. For example, many companies are developing drugs against kinases, such as p38 and JNK MAPK (23). However, these drugs are burdened with toxicity and side effects. Some bioactive natural substances, such as glycyrrhizic azid, can enhance the efficacy of GCs by inhibiting p38 (155). Another therapeutic option for treating GCR is to reverse the GR defect at the level of GR physiology itself. For example, selective activation of HDAC2 with theophylline and PI3Kδ inhibitors can reverse GCR (156). Clinical trials with low-dose theophylline combined with GCs are currently ongoing. PI3Kó inhibitors are already in clinical development for the treatment of severe asthma, but they might be combined with GC therapy in the future.

As TNF is an important mediator in the pathogenesis of asthma, studies have investigated the use of TNF antagonists in asthma patients. A small initial study showed a beneficial effect of an anti-TNF therapy (131), whereas a larger study later showed less promising results (157). However, the effects on steroid resistance were never assessed, as these studies defined their study population by the severity of disease and not by the degree of reversibility after steroid

administration. It is important to do so, because most immune disorders are heterogeneous, with distinct phenotypes and with different pathogenic mechanisms requiring different therapeutic approaches. This explains why some patients with an inflammatory disorder are glucocorticoid resistant. It should be kept in mind that not all GC insensitive patients suffering from different pathologies are insensitive for the same reason. Even for a certain disease, not all patients with GCR are necessarily insensitive to GCs for the same reason. Consequently, some drugs will be more effective than others in each subgroup of patients. Therefore, it is important to identify the different inflammatory phenotypes in inflammatory diseases and the cytokine profile that reflects the subgroups of patients that are steroid resistant. The identification of biomarkers will allow the development of novel and effective personalized therapeutic approaches.

Previous studies have shown that monoclonal antibodies against inflammatory mediators have numerous adverse effects related to their specific targets. For example, the use of p38 MAPK inhibitors may not be so effective due to redirection of the inflammatory response to the JNK pathway, which could also disrupt GR function. Therefore, combined inhibition of p38 and JNK might be more effective compared to mono-targeting strategies and was shown already to reverse GC insensitivity (158). Consequently, the use of these combinatorial drugs in conjunction with GCs may be warranted (159). Immune diseases are indeed mostly driven by multiple cytokines and inflammatory mediators, and multiple inflammatory pathways might be involved in the development of GCR. As each of these is a potential therapeutic target for the reversal of GCR, mono-therapy might not be effective.

CONCLUSIONS AND FUTURE PERSPECTIVES

We have clearly documented that GR function can be negatively modulated under pro-inflammatory conditions (Figure 1; Table 1). A more detailed understanding of the molecular mechanisms of GR action and inaction may reveal new drug targets that could be exploited to sensitize resistant diseases to the anti-inflammatory effects of GCs. In addition, there is a need for specific biomarkers to identify patients who are likely to benefit from new therapies. Most current therapeutic strategies are based on GC-sparing agents. However, the development of a GC-sensitizing agent, thus reversing the mechanisms responsible for the defect in GC action, could also enable effective therapy of GCR patients, if given in combination with GCs.

Recent advances in the understanding of the interaction between GR and pro-inflammatory signaling pathways indicate that inflammation can also exert positive effects on GC signaling. Exposure of different cell types to pro-inflammatory cytokines results in increased GR expression (160, 161), which suggests enhanced GC responsiveness. Similarly, cytokines have been shown to increase the bioavailability of GCs by increasing the activity of 11β-HSD1 (162). In this way, the pro-inflammatory environment might enhance the anti-inflammatory responses mediated by GR. On the other hand, GC signaling can also have a pro-inflammatory character, for example, GCs positively regulate the pathogen recognition receptors NLRP3 (163) and TLR2 (164). The latter is the result of cooperative binding of GR with other transcription factors to induce transcription. In this way, in addition to anti-inflammatory genes such as Tnfaip3 (A20), multiple pro-inflammatory genes are synergistically upregulated (165). For example, serpinA3, an acute phase protein involved in several inflammatory diseases, is coregulated after simultaneous challenge with GCs and TNF (41). It is possible that inflammatory mediators enhance their own potential by stimulating these GR-mediated actions in this way. But in these studies GCs were administrated before or simultaneously with the inflammatory stimulus. It is likely that this results in a different transcriptional outcome compared to the initiation of inflammation before GR activation, the latter of which relates more to the clinical situation. In addition, the group of Cidlowski hypothesized that the initial increased GC responses driven by an inflammatory response are required to stimulate the inflammatory state to ensure clearance of pathogens (165). Generally, it is an imbalance in the different effects of cytokines on GR action that is responsible for GCR.

FIGURE LEGEND

Figure 1: Mechanisms of glucocorticoid (GC) action and the impact of cytokines. GCs are produced by the hypothalamic-pituitary (HPA) axis and bound by corticosteroid binding gobulin (CBG) in circulation. Intracellularly, GCs are converted to their bioactive form by 11beta-hydroxisteroid dehydrogenase type 1 (11β-HSD1), after which they bind the glucocorticoid receptor (GR). The expression of GR from its gene, *Nr3c1*, is regulated by many transcription factors (X and Y), as well as by GCs via the presence of a negative GC response element. Upon binding of GCs to their (GR), the GR chaperone complex, including HSP90 and p23, is modified and GR is subjected to posttranslational modifications, both of which assist GR in its translocation to the nucleus where it exerts its functions. The anti-inflammatory actions of GR are mediated by both transcriptional induction as repression mechanisms, instructed by coactivators and corepressors, repectively. However, pro-inflammatory cytokines (red dots) negatively interfere with GR function by blocking all levels of the physiological activities of GCs/GR. P, phosphorylation; NO, nitrosylation; Ac, acetylation; SUMO, sumoylation.

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Table 1: Impact of cytokines and downstream inflammatory mediators on GR function.

The actions glucocorticoid receptor (GR) can be negatively influenced by the pro-inflammatory environment, in many different ways, of which most are listed below. Note that it is not always known which inflammatory cytokines are involved in the effects impairing GR function (*not known* in the column 'Cytokine'). On the other hand, the specific mechanism by which cytokines negatively affect GR actions is sometimes not studied yet (not known in the column 'Mechanism') .

Cytokine	Mechanism	Cell type/tissue	Reference
Altered ligand availability			
IL1 and TNF	Activation of HPA axis	Cerebral cortex	(34)
IL6	Inhibition of CBG secretion	HepG2 cells	(36)
Cytokines	Increased catabolism of CBG	Granulocytes	(35)
TNF	Reduced CBG levels	Serum	(37)
IL1β and TNF	Increased expression and activity of 11βHSD1	MG-G3 cells + primary osteoblasts	(38)
		Glomelural mesangial cells	(39)
TNF	Reduced expression and activity of 11βHSD2	HepG2 cells	
		MG-G3 cells + primary osteoblasts	(38, 40)
TNF	Increased MDR activity	PBMCs	(46)
Reduced ligand binding			
IL2 and IL4	P38 MAPK activation	PBMCs	(83)
Not known	GR nitrosylation	L929 cells	(14)
		Lung and spleen	(49)
IL1	*Not known*	Reuber hepatoma cells	(54)
IL13	*Not known*	Monocytes	(80)
Not known	P38 MAPK activation	HeLa cells	(86)
Reduced glucocorticoid receptor levels			
IL1α	*Not known*	Rat hepatoma cells	(54)
TNF	*Not known*	U937 cells	(5)
		Hepatocytes	(56)
IL2 and IL4	Increased GRβ/GRα ratio	PBMCs	(62)
IL17 and IL23	Increased GRβ/GRα ratio	PBMCs	(64)
TNF and IL1	Increased GRβ/GRα ratio	HeLa and CEMC7 cells	(6, 10)
Cytokines	Degradation via proteasome	*Not known*	(6, 10)
Reduced GR levels by increased glucocorticoids via homologous downregulation			

Cytokines	miRNA-124	Brain	(70)
Cytokines	ACTH regulates miRNA-96, -101a, -143, -433, which target GR	*Not known*	(69)
IL1, IL2 and IL6	Increased CRH and ACTH levels	Paraventricular nucleus & corticotrophic cells	(166, 167)
Impaired glucocorticoid receptor nuclear translocation			
IL13	*Not known*	Monocytes	(80)
IL1	Activation of p38	Fibroblasts, macrophages	(78, 82)
IL2 and IL4	Activation of p38	PBMCs	(83)
Altered glucocorticoid receptor posttranslational modifications			
IL2 and IL4	GR phosphorylation	PBMCs	(83)
TNF and IFNγ	Reduced Ser211 phosphorylation	Airway smooth muscle cells	(84)
Cytokines	Phosphorylation of Ser226 + sumoylation	COS-7 and A549 cells	(13, 85)
Negative effects on GR function by transcription factor activation			
TNF	MEK-1/ERK cascade	HaCaT cells	(96)
IL2 and IL4	P38 MAPK activation	PBMCs	(83)
IL2	STAT5 and JAK3 activation	T cells	(97)
TNF	NFκB and AP1 activation	*Not known*	(153)
TNF and IFNγ	IRF1 activation	ASM cells	(110, 111)
Abnormal chromatin environment			
TNF and IFNγ	Decreased GRIP1 availability	ASM cells	(110)
TNF	Decreased SRC1 and 2 expression	UtSM cells	(112)
IL17	Reduced HDAC2 activity	Bronchial epithelial cells	(117)

Abbreviations: HPA, hypothalamic-Pituitary-Adrenal; CBG, Corticosteroid-Binding Globulin; TNF, Tumor Necrosis Factor; IL, Interleukin; PBMC, peripheral Blood Mononuclear Cell; GR, Glucocorticoid Receptor; MAPK, Mitogen-Activated Protein Kinase; HSD, Hydroxy Steroid Dehydrogenase; MDR, MultiDrug Resistance; ACTH, AdrenoCorticoTrophic Hormone; CRH, Corticotrophin Releasing Hormone; ASM, Airway Smooth Muscle; UtSM, Uterine Smooth Muscle; HDAC, Histone DeAcetylase; SRC, Steroid Receptor Coactivator, IFN, Interferon, AP, Activator Protein; ERK, extracellular signal-regulated kinase; Ser, Serine; miR, microRNA.

Author Biography

Lien Dejager finished her PhD in Biotechnology from the University of Ghent in 2010 under the promotership of Prof. Claude Libert, IRC, VIB. Afterwards she became a postdoctoral researcher at FWO-Vlaanderen in the same group. Her major research interests are elucidating the anti-inflammatory mechanisms of glucocorticoids and the mechanisms underlying glucocorticoid resistance, aiming to design more efficient glucocorticoid-based therapies.

Sofie Vandevyver is a Post-doctoral researcher in Prof. Dr. Claude Libert's Lab in the Inflammation Research Center, University of Ghent. She obtained a degree in Biochemistry from the 'Hogeschool West-Vlaanderen', after which she obtained a degree in Biotechnology from the University of Ghent. She started her PhD in the IRC in 2009 after obtaining a fellowship from the Flemish Government Agency for Innovation by Science and Technology (IWT) and finished in 2013. Her research is mainly focused on unraveling the mechanisms of Glucocorticoid Receptor Function, predominantly transactivation, and Glucocorticoid Receptor Resistance in inflammation.

Ioanna Petta is a PhD student in a shared project between the Lab of Mouse Genetics in Inflammation (MGI) of Prof. Claude Libert in the Inflammation Research Center (IRC) and the Cytokine Receptor Lab (CRL) of Prof. Jan Tavernier, University of Gent-VIB. She obtained her Bachelor degree in Biology from the University of Patras (Greece), after which she conducted her Master in Biotechnology in the same University. She started her PhD in 2010 after obtaining a fellowship from the VIB for international PhD students. Her research is focused on the identification and functional analysis of novel interaction partners of the Glucocorticoid receptor in inflammation.

Claude Libert is a full professor at University Ghent and a Group leader at VIB. He obtained his PhD in 1993 and did a postdoc in the IRBM, Rome, Italy, with Valeria Poli and Gennaro Ciliberto during 1994-1995. In 1997 he became VIB group leader. His interest lies mainly in sepsis and other acute inflammatory conditions. His group wants to find new insights in sepsis and is focusing on TNF, MMPs and glucocorticcoids to achieve their goals.

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