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The increasing complexity of glucocorticoid receptor signaling and regulation

Sofie J. Desmet[a], Ilse M. Beck[b], Nadia Bougarne[a], Dorien Clarisse[b], Julie Deckers[a,c], Dariusz Ratman[a], Jan Tavernier[a] and Karolien De Bosscher[a*]

Received: 02.10.2013 *Accepted: 20.01.2014* *Published: 08.05.2014*

[a] *Cytokine Receptor Laboratory, VIB Department of Medical Protein Research,VIB, Ghent University, Albert Baertsoenkaai 3, B-9000 Ghent, Belgium*
[b] *Laboratory of Experimental Cancer Research (LECR), Department of Radiation Therapy & Experimental Cancer Research, Ghent University Hospital, De pintelaan 185, Building 1P7, B-9000 Ghent, Belgium*
[c] *VIB Department for Molecular Biomedical Research, VIB, Ghent University, 'Fiers-Schell-Van Montagu' building, Technologiepark 927, B-9052 Ghent (Zwijnaarde), Belgium*

** Corresponding author. Tel: +32 92649363; fax +32 92649490 karolien.debosscher@vib-ugent.be sofie.desmet@vib-ugent.be*

Abstract

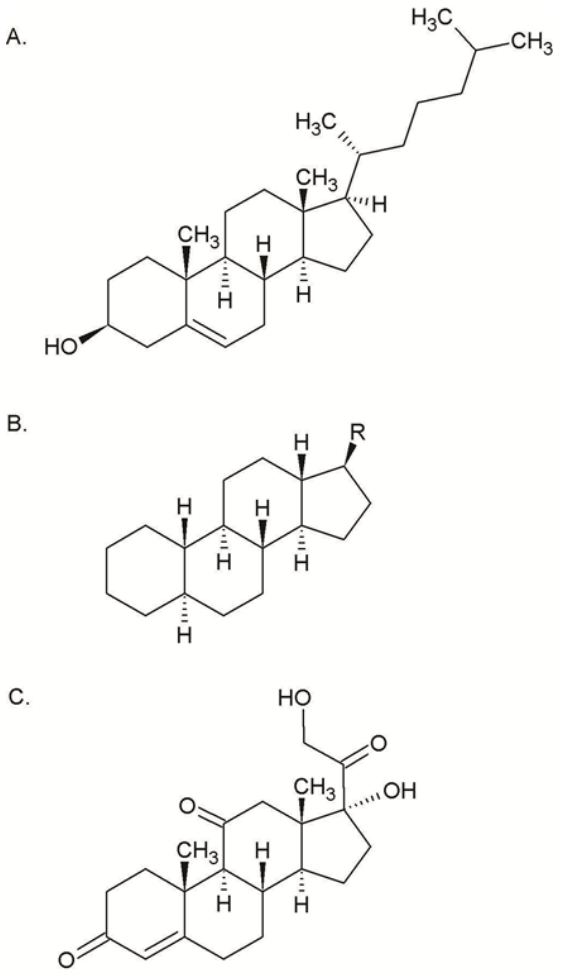
Glucocorticoids, although being one of the eldest drugs in the clinic and despite their widespread usage for the treatment of inflammatory and immune disorders and cancer, have not yet come of age when it comes to a full understanding of how they work. The majority of the biological actions of glucocorticoid hormones are explained by a wide diversity in the cellular action mechanism of the hormone-activated Glucocorticoid Receptor (GR). All molecular mechanisms described in the current overview are not only complex, exhibiting an astonishing degree of gene- and tissue-specificity, but on top of this they are also non-exclusive. This layering of mechanisms makes it extremely difficult for researchers to extract the crucial pieces of information that would assist in a rational design of drugs with an improved therapeutic profile, i.e. a satisfying and maintained therapeutic response in the absence of the many incapacitating glucocorticoid-associated side effects, such as diabetes, osteoporosis, muscle wasting, depression etc. In direct correlation with increased glucocorticoid usage as observed in the clinic, the impetus and desire to reveal all of these mechanisms -and most importantly, to try to integrate them in a sensible manner for the sake of finding better alternatives- has never been stronger.

Keywords: *Glucocorticoid, Glucocorticoid Receptor, mechanism, transcription, signal transduction, regulation*

INTRODUCTION: THE CLINICAL IMPORTANCE OF GLUCOCORTICOIDS

In the late 1940s, a rheumatoid arthritis patient *miraculously* recovered from his symptoms, albeit temporarily, after treatment with cortisone. Following this observation, Hench, together with Kendall and Reichstein, received the Nobel Prize for their findings on the adrenal glucocorticoid hormone in 1950 (1). This hormone is derived from cholesterol, has a typical steroidal structure (Figure 1) and is secreted by the zona fasciculata of the adrenal gland. Glucocorticoids (GCs) play a pivotal role in various biological processes, such as metabolism, reproduction, development, inflammatory reactions and stress responses. GCs are regulated in a circadian and stress-associated manner with the goal to maintain various metabolic and homeostatic functions that are necessary for life. The synthesis and release of natural glucocorticoids is subject to a circadian and ultradian rhythm, controlled by the hypothalamus-pituitary-adrenal axis

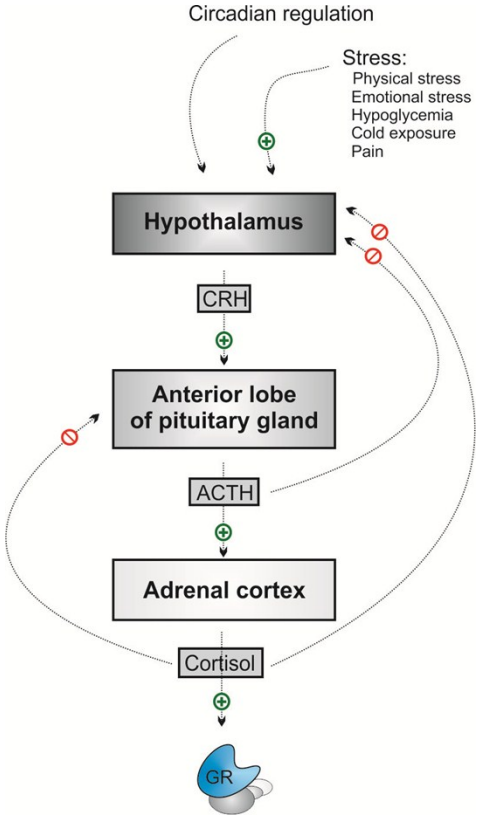
Figure 1. Steroidal structures. A. Chemical structure of cholesterol. B. Basic steroidal structure in which 'R' represents any side chain. C. Chemical structure of cortisone



(HPA-axis, Figure 2) (2,3), with the lowest levels reached late night-early morning. Hence, sufferers from asthma typically experience their fiercest attacks around that time (4).

Following the discovery of the therapeutic potential of cortisone, a wide range of synthetic derivatives have since revolutionized clinical medicine. Despite the wide range of side effects caused by pharmacological dosages of GCs, therapies based on these GCs are currently still the most effective treatment for all kinds of inflammatory and immune disorders. Examples are asthma, rheumatoid arthritis, inflammatory bowel disease, autoimmune diseases and transplant rejection. With regard to inflammation, it is well accepted that the therapeutic action of GCs is mainly achieved by the dampening of pro-inflammatory signal transduction pathways and in consequence, the effective inhibition of multiple activated pro-inflammatory genes. Additionally, due to their role in the induction of apoptosis (programmed cell death) and because of their anti-angiogenic and anti-emetic actions, GCs can be applied as a component of chemotherapy for the treatment of a number of cancers (5–7). Although also used to treat solid tumors (8), GCs constitute an important

Figure 2. Hypothalamus - Pituitary - Adrenal (HPA)-axis. Regulation of glucocorticoid, i.e. cortisol, production and release.
Abbreviations: CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; GR, glucocorticoid receptor

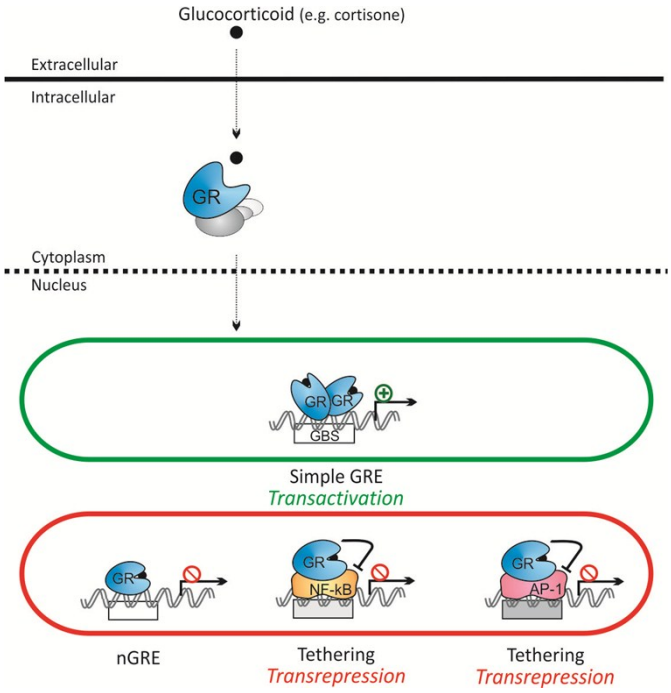


part of the treatment of lymphoid malignancies (7,9). As a consequence of their broad action, GCs also cause a plethora of side effects, such as osteoporosis, muscle atrophy, hypertension, growth suppression in children, and abnormalities in glucose and fat metabolism, which limit the use of GCs as a robust, long-term therapy (5,7). In addition, the therapeutic effects decrease during treatment due to the gradual onset of glucocorticoid resistance, further limiting their action spectrum. The conundrum of GC resistance also poses a considerable problem to the scientific community, because the underlying mechanisms of GC resistance seem divergent, with a cell-type specific and highly controversial component (6,10,11).

THE COMPLEXITY OF A WIDESPREAD GLUCOCORTICOID RECEPTOR BINDING ONTO GENOMIC REGIONS

GCs act via the Glucocorticoid Receptor (GR), a ligand-dependent transcription factor that belongs to the thyroid/steroid nuclear receptor superfamily (3,12). Both genomic and non-genomic mechanisms for the action of glucocorticoids have been described (2). Due to their lipophilic nature, GCs can diffuse freely through the cell membrane and bind to the cytoplasmic GR (Figure 3). Ligand binding elicits a conformational change in the receptor, followed by the dissociation of heat shock proteins and nuclear translocation. Heat shock proteins serve as chaperoning proteins, with Hsp90 and Hsp70 as the most important ones, enabling a net residence of the unliganded, inactive receptor in the cytosol. Once the ligand-bound receptor is in the nucleus, the expression of GC-responsive genes can be influenced in a positive or negative manner. Positive control is primarily mediated by transactivation, a process in which the ligand-activated, homodimeric GR binds to glucocorticoid response elements (GRE) via its centrally located DNA-binding domain (see chapter 3) (13,14). GREs are inducible enhancer elements in the promoter region of GC-responsive genes and typically consist of one or more GR binding sequence (GBS), with the consensus sequence 5′-AGAACANNNTGTTCT-3’ (15), and eventual binding motifs for non-GR transcriptional regulatory factors (16–18). Of note, GR target genes have been identified for which the GBS deviates from the above consensus sequence, which contribute to the diversity in GR signaling (2,16). It is important to point out a recent change in GRE nomenclature and to clearly make the distinction between the above described GREs and so-called GR binding regions (GBRs) in the genome, detected using genome-wide chromatin immunoprecipitation approaches (ChIPseq) and retrieved in a context-dependent manner (e.g. varying dependent on the cell type) (Figure 4) (16). Intriguingly, a recent study of the Yamamoto team (16) elegantly showed that GBSs can dictate structural changes at the DNA-binding interface that are subsequently translated into changes in the GR dimerization interface, a phenomenon

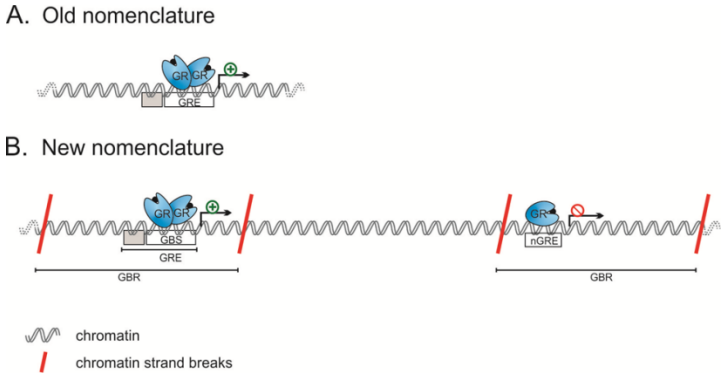
Figure 3. Activation of GR and GR-regulated transcription
Glucocorticoids (GCs), such as cortisol, can diffuse across the plasma membrane. In the cytoplasm these GCs bind into the ligand-binding pocket of the glucocorticoid receptor (GR). Upon ligand binding, the GR dissociates from its cytoplasmic chaperoning molecules and travels to the nucleus where it can affect gene transcription via multiple mechanisms. The binding of homodimeric GR onto the GR-binding sequence (GBS) of a GC response element (GRE) results in enhanced transcription. Alternatively, inhibition of transcription is attained via binding of monomeric GR onto a negative GRE (nGRE) or binding of monomeric GR onto the transcription factors NF-κB or AP-1. The latter tethering mechanisms are called transrepression.



that is further transmitted into a distinct impact on the transcriptional outcome. Other regulating mechanisms for gene expression have also been described, such as binding of the GR to negative GREs, resulting in inhibition of gene expression (19). This mechanism however represents a minority within the whole of GR target genes. GR can also negatively interfere with the action of other activated transcription factors, such as the pro-inflammatory Nuclear Factor-kappaB (NF-κB) and Activator Protein-1 (AP-1). This mechanism, in which protein-protein interactions are involved, is referred to as transrepression (see chapter 7) (14).

A general assumption is that the unwanted effects are mainly caused by GR-mediated transactivation, while transrepression of various pro-inflammatory genes accounts for the anti-inflammatory potential of GCs. However, not all the side effects can be predictably prevented by an uncoupling of transactivation and transrepression. Exemplary herein is the Hypothalamic-Pituitary-Adrenal (HPA) axis suppression, which relies on transrepression and would thus be sustained using transrepression-favouring GR ligands (20,21). And, vice versa, some important anti-inflammatory effects of GCs are caused by upregulating

Figure 4. Graphical illustration of recent changes in GR-DNA interaction nomenclature
While in the old nomenclature (A), the glucocorticoid response element (GRE) is preserved for the palindromic 15bp sequence onto which homodimeric GR actually binds, the new nomenclature (B) has a wider understanding for a 'GRE' and lets it encompass the 15bp glucocorticoid receptor-binding sequence (GBS), as well as eventual adjacent regulatory elements. The glucocorticoid receptor-binding regions (GBRs) are DNA sequences, identified by genome-wide chromatin immunoprecipitation analyses, that show a positive signal for GR binding within the cleaved DNA strand.
Abbreviations: GRE, glucocorticoid response element; GBS, glucocorticoid receptor-binding sequence; GBR, glucocorticoid receptor-binding region



anti-inflammatory genes via transactivation, for instance the gene encoding the GC-Induced Leucine Zipper (GILZ) protein, DUSP1 (dual-specificity phosphatase 1), or the Inhibitor of NF-κB (IκBα) protein (20).

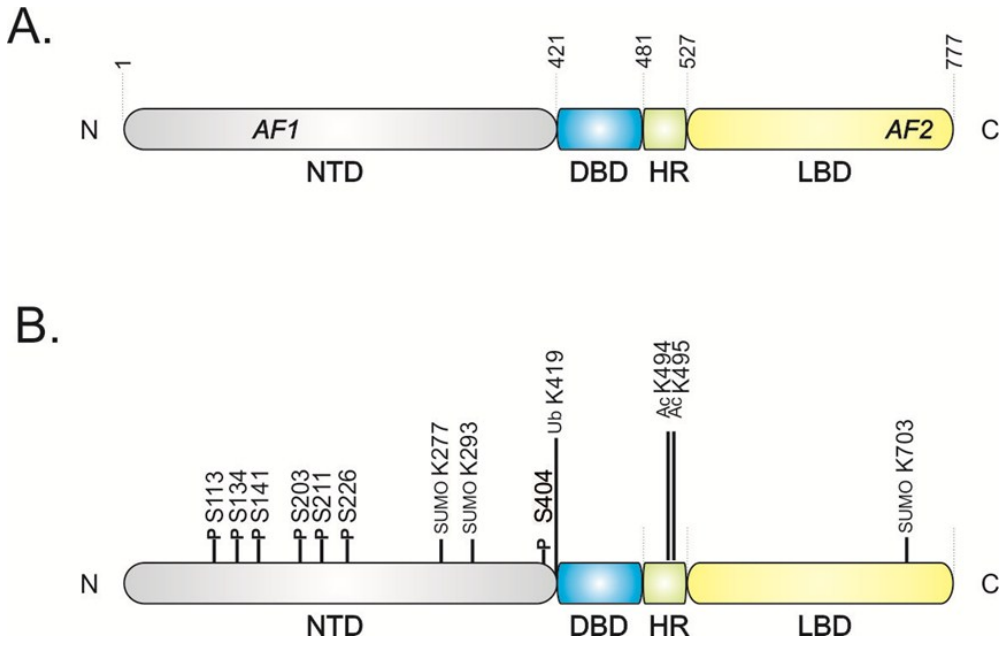
THE COMPLEXITY OF GLUCOCORTICOID RECEPTOR ISOFORMS

GR is a modular protein composed of an N-terminal transactivation domain (NTD), a central DNA binding domain (DBD), a hinge region (HR) and a C-terminal ligand binding domain (LBD) (Figure 5A). The NTD comprises a first transactivation domain, also called activation function 1 (AF1), which plays an important role in the recruitment of molecules necessary for the initiation of transcription. The DBD contains two zinc finger motifs that recognize and bind the GBSs. Furthermore, this domain contains sequences that are important for receptor dimerization, nuclear translocation and binding to other transcription factors. From an evolutionary perspective, the DBD is the most conserved domain among the 48 members of the nuclear receptor superfamily. The LBD plays an essential role in the ligand-induced activation of the GR and contains a second transactivation domain, called AF2. This transactivation domain is, in contrast to AF1, ligand-dependent. The LBD also contains sequences involved in nuclear translocation, binding to heat shock proteins, receptor dimerization and interaction with coregulators. Coregulators are positive (coactivators) or negative (corepressors) regulatory proteins that are necessary for the transcriptional activity of the GR and are recruited by the receptor

Figure 5. Organization of the glucocorticoid receptor isoform hGRα-A

A. Structural organization of the glucocorticoid receptor isoform hGRα-A. B. Post-translational modifications of hGRα-A (see chapter 4)

Abbreviations: NTD, N-terminal domain; DBD, DNA-binding domain; HG, hinge region; LBD, ligand-binding domain; AF, activation function; P, phosphorylation; SUMO, sumoylation; Ub, ubiquitinylation; Ac, acetylation.



through protein-protein interactions (22). Finally, there is a hinge region between the DBD and LBD, which gives structural flexibility to the GR. Because of this flexibility, a single receptor dimer can interact with multiple GREs (3,23).

Multiple isoforms of the human GR are generated by alternative RNA splicing and alternative translation initiation. The most common isoforms are GRα and GRβ, produced by alternative splicing of the last exon, exon 9, of the human NR3C1 gene. GRα is ubiquitously expressed and is the classic, ligand-dependent form of the receptor. GRβ, in contrast to GRα, does not bind to GCs and is transcriptionally inactive. Instead, it can have a dominant negative effect on the transcriptional activity of the GRα isoform and is therefore associated with GC resistance (2). Additional splice variants are detected in specific tissues and some have been associated with certain diseases, exemplified by the hGRγ isoform that has been detected in childhood acute lymphoblastic leukemia. Most of these variants are associated with a lower transcriptional activity. Besides these splice variants, there are numerous variants generated by alternative initiation, such as GRα-B, C1-3 and D1-3. These variants can display distinct expression, transactivation and transrepression patterns. As such, alternative splicing and translation initiation are additional mechanisms that regulate the expression of GC-target genes and can explain tissue- and disease-specific effects (2,3,24).

THE COMPLEXITY OF POST-TRANSLATIONAL MODIFICATIONS

An additional regulatory level impacting the transcriptional activity of GR is achieved by post-translational modifications (PTM), including phosphorylation, acetylation, ubiquitinylation and SUMOylation (Figure 5B). These modifications can influence numerous factors, such as subcellular localization, protein half-life, coregulator recruitment, ligand or DNA binding, and as a result the overall transcriptional activity of the receptor. The PTM of the receptor can be considered as a unique "code", of which the composition depends on signals from both outside and inside the cell (25,26).

One of the best characterized modifications is phosphorylation. When phosphorylation occurs, a phosphate group is associated to a protein by a specific enzyme, namely a kinase. This association can be reversed via the action of other enzymes, called phosphatases. The GR has a low basal phosphorylation status and gets hyperphosphorylated following binding of an agonist. Phosphorylation of the serine residue at position 211 (S211) is considered as a hallmark for its transactivation potential (25,26). Ligand binding also induces acetylation of GR; a process in which an acetyl group is introduced into lysine residues of the protein. These acetyl groups are subsequently removed by deacetylases, e.g. the enzyme histone deacetylase 2 (HDAC2) that not only targets histones but also other proteins. The latter process was reported as a crucial mechanism allowing GR to inhibit the activity of the pro-inflammatory NF-κB (3,27). A third possible modification is ubiquitinylation, an energy-dependent process in which an ubiquitin is transferred from an ubiquitin-activating enzyme (E1) to an ubiquitin-conjugating enzyme (E2) and finally to the target protein by an E3 ligase enzyme (28).

Different PTMs can also influence each other. For example, specific phosphorylation events trigger polyubiquitinylation and subsequent degradation, which ensures a rapid turnover of the receptor and thereby a decrease of receptor activity. This outcome is not universal and cannot be extrapolated to other steroid receptors. Indeed, in contrast to GR, ubiquitinylation of the estrogen receptor (ER) is even a requirement for continued transcriptional activity (28). Finally, SUMOylation refers to the covalent attachment of SUMO (Small Ubiquitin-related MOdifier) proteins via enzymes similar to those mediating ubiquitinylation. SUMOylation sites in the GR are located in the N-terminal AF-1 and LBD, and the effect of this modification is highly dependent on the promoter context (29). Recently, this type of modification on a lysine in the LBD was found to confer a positive effect on GR-mediated transcription, in contrast to N-terminal domain SUMOylation. Hence, inhibitory and stimulatory SUMO sites are present in the GR and intriguingly, at higher SUMOylation levels the stimulatory site becomes dominant over the others (30). Appreciably, it

becomes extremely difficult to predict the behavior of GR when dissecting only a subset of PTMs, and rather, it is the complete set of modifications that co-determines in which direction transcriptional events are driven.

THE COMPLEXITY OF REGULATION BY COFACTORS

In 1995 the first nuclear receptor coactivators were cloned (31,32), soon after followed by corepressors (33,34). Nowadays, it has become clear that the delineation coactivator-corepressor is not as sharp as originally assumed, and that coactivators can turn into corepressors, depending on the cellular context and identity of the genes being targeted (35,36). Coactivators/corepressors, or taken together as so-called coregulators, bridge GR with the transcription initiation complex and influence the activity of RNA polymerase II. The conformational change elicited by ligand binding results, next to a dissociation of heat shock proteins and nuclear translocation, in the formation of various interaction surfaces for multiple regulatory proteins, the so-called coregulators (7,37,38).

At the molecular level, the LBD of GR consists of α-helices which form a hydrophobic cavity. AF2, a helix at the C-terminus of the LBD, is essential for ligand-dependent transcriptional activation. This activation helix takes different positions depending on the presence or absence of ligands. Binding of an agonist, which is an activating ligand, leads to adoption of a so-called 'charge clamp' conformation. This configuration allows for the recruitment of coregulators, mainly coactivators, which contain the LXXLL consensus sequence. The leucine residues of this consensus sequence interact with the hydrophobic cavity formed by the LBD of GR. Many coactivators contain multiple LXXLL motifs, which may be used in a nuclear receptor-specific manner, thereby allowing a modulation of the efficiency of the coactivator function. Corepressors interact with unbound or antagonist (a suppressing ligand)-bound nuclear receptors via the longer sequence LXX I/H IXXX I/L, also called nuclear receptor corepressor (CoRNR)-box, that binds to the same hydrophobic cavity as the LXXLL motifs. However, this binding mode is not possible when the activation helix adopts a 'charge clamp' configuration in response to agonist binding. In conclusion, binding of an agonist diminishes the affinity of the receptor for CoRNR-box-containing corepressors and enhances it for LXXLL-containing coactivators. Some corepressors however can also be recruited in a ligand-dependent manner by the presence of LXXLL motifs, and can thus compete with coactivators. An example hereof is the ligand-dependent corepressor (LCoR) (34).

It is not a single coregulator that does the job in initiating and perpetuating gene expression or vice versa. It is neither the sequential activity of one coregulator at a time, but rather a dynamic complex of coregulators that collaborate, and of which the composition can vary substantially. For example, coactivator complexes typically consist not only of adaptor proteins (e.g.

p160 family members) but also of various histone-modifying proteins (see next chapter), of which the role is to relax or condense the chromatin. Coregulator complex composition associated with GR, as well as the activity of various coregulators in the complex depends on the tissue type, the identity of the GR-activating ligand and the specific target gene promoter structure (see above). Not only GR as a transcription factor is subject to post-translational modifications, influencing its activity and stability, but also the coregulators themselves, down to the level of the basal transcription factors and RNA polymerase II (34,37,39). All these on/off switches at various levels need to be integrated and translated to a logic transcriptional outcome, of which the directionality (gene active or inactive) is often hard to predict, and the extent or duration of activation or repression even harder.

In addition to the traditional ligands, DNA itself can be regarded as a sequence-specific allosteric ligand of the GR, a concept which was raised a long time ago, but for which more and more evidence has been gathered in the past decade (16,40,41). The GR-responsive DNA sequence can affect the configuration and therefore the activity of the receptor via the recruitment of specific coregulators. The GBSs may differ between promoters in sequence, number and position relative to the transcription start site. As a consequence, both the characteristics of the binding site and the ligand can define the specific assembly and function of coregulators via alterations in the receptor structure and, subsequently, influence the specificity and magnitude of the response of the gene in question (16,23,25,40,41).

THE COMPLEXITY OF EPIGENETIC MECHANISMS AND THE CHROMATIN LANDSCAPE

DNA methylation and histone modifications are epigenetic mechanisms that 'tag' genes, hereby controlling genome functionality at different levels. The environment can additionally modulate this 'tagging' process, a phenomenon which is believed to contribute to the onset of diseases (42). The overall function of GR is regulated by various factors including chromatin structure, epigenetics, genetic variation and the temporal pattern of glucocorticoid hormone secretion (43). In epigenetics, DNA methylation is a modification most often associated with chromatin condensation, transcription factor binding occlusion and gene silencing (5,34). This modification was demonstrated at the GR promoter in specific brain regions and subsequently shown to influence expression of the GR gene. The reduction of central GR expression coincided with resistance to glucocorticoids. Interestingly, it has also been shown that deprivation of maternal nurturing correlates with an increase in DNA methylation of the GR promoter in the hippocampus. Even more intriguing, this methylation pattern could be passed on to further generations (44). Another example that nicely illustrates a dynamic cross-talk between epigenetic

changes and environmental cues or at least individual experience, was found in suicide victims with a history of child abuse, which display elevated GR promoter methylation in their post-mortem hippocampi (45).

It was mentioned above that GR does not act on its own but instead, makes part of multifactorial regulatory complexes. Engel and Yamamoto (46) studied how GR and the coregulator Brm, an ATPase subunit of the Swi/Snf chromatin remodeling complex, would affect each other's activity and occupancy on the genome. Hereto, the effect of a Brm knockdown was monitored for several GR target genes in cells treated or not with GCs. It appeared that GR occupancy on DNA and its activity were differentially changed at specific primary GR target genes, both activated and repressed. Their results support multiple distinct patterns of an interdependence of GR and Brm. So studying only these two variables as paradigms for a combinatorial regulation within regulatory complexes already reveals marked functionally distinct assemblies (46).

To conclude, both genetic (e.g. small nuclear polymorphisms) and epigenetic variations will contribute to glucocorticoid sensitivity and responsiveness. How widespread the occurrence of these variants among the general population is, is as of yet not clear.

THE COMPLEXITY OF PROTEIN-PROTEIN INTERACTIONS BETWEEN THE GLUCOCORTICOID RECEPTOR AND OTHER PROTEINS

Next to interactions with coregulators and chromatin-modifying enzymes, the GR binds to a wide range of other transcription factors, including the pro-inflammatory AP-1, NF-κB and Signal Transducers and Activators of Transcription (STAT) (14,47). These protein-protein interactions generally result in the suppression of the transcriptional activity of the targeted factor, but depending on the context they can also enhance gene expression. In contrast to the binding of GR to classical or negative GREs, this mechanism, called transrepression, does not involve DNA-binding of the receptor, but binding to the DNA-bound transcription factor (tethering) or occasionally removing the transcription factor from its binding site (squelching). This direct interference, resulting in a protein synthesis-independent inhibition of cytokine gene expression, is considered the primary anti-inflammatory action displayed by the GR (48–50). Contradictory, in the case of the pro-inflammatory stimulus IL-1β, transactivation of anti-inflammatory genes appears to represent the major anti-inflammatory mechanism used by the GR. Examples of such anti-inflammatory proteins are DUSP1, which inactivates all three major MAPK pathways, and GILZ that among other actions represses both AP-1 and NF-κB signaling. Consequently, it is important to realize that glucocorticoids have distinct effects on different inflammatory responses, and that not all inflammatory genes are repressed by these hormones. This differential

regulation may allow preserving necessary aspects of host defense or feedback regulation (50).

Transrepression typically takes place in both directions, in which the GR-mediated gene expression is reciprocally suppressed by the same transcription factor (25). Understanding the molecular basis for transrepression is ongoing, as it becomes clear that not one mechanism is responsible, but rather several mechanisms can be applied depending on context-specific components. One example of such a mechanism is the recruitment of the GR-interacting protein-1 (GRIP-1), which acts in this context as a corepressor, when the receptor tethers to the DNA-bound AP-1 or NF-κB transcription factor. In addition, this complex can deploy different modes of action and interfere at different steps of the transcription cycle to repress inflammatory gene expression (51).

A special case of an interaction between GR and another protein is the crosstalk with the transcription factor Peroxisome Proliferator-Activated Receptor (PPAR) α. This lipid-activated nuclear receptor not only tethers to GRE-bound GR, which results in a gene-specific modulation of GR's transcriptional activity (Ratman *et al.*, manuscript in preparation), but also enhances the GR-mediated transrepression of NF-κB-driven gene expression. Via these actions, PPARα can circumvent GRE-mediated side-effects and additively represses pro-inflammatory cytokine expression (52,53).

THE COMPLEXITY OF NON-GENOMIC GC ACTIONS

Pleiotropicity and diversity in the function of glucocorticoids is also reached by the so-called non-genomic action mechanisms. These mechanisms include for example the effect of glucocorticoids on the phosphorylation and activation of MAP kinases. Depending on the cell type, it has been shown that glucocorticoids can inhibit cytokine-induced JNK, ERK or p38 kinases by blocking the phosphorylation step that is needed to activate these kinases (reviewed in (14)). GR and JNK have further been demonstrated to interact physically and JNK can also phosphorylate hGR at position S226, inhibiting the transcriptional activation of GR (reviewed in (25)). Other non-genomic mechanisms of glucocorticoids include receptor-independent observations, deemed responsible for e.g. the rapid inhibitory effects on human neutrophil degranulation at the cellular level (54), and activities via a membrane-bound GR supporting the occurrence of rapid anti-inflammatory effects (55–57). In a continued attempt to resolve the direct and indirect effects of GCs, the focus has recently shifted to the mitochondria as a newly emerging area of intense GC research. It is suspected that mitochondria may also be under GC control since GR is present in mitochondria, and GREs reside in the mitochondrial genome (58,59). It is currently under investigation which of the two main possible mechanisms of GC regulation predominates: either a direct action on mitochondrial DNA and oxidative phosphorylation genes, or an indirect effect through the interaction with

nuclear genes (59). How GCs can affect the broad spectrum of mitochondrial functions is an exciting novel field of research and results that follow will hopefully increase our understanding of yet another complexity in GR's action mechanism.

CAN DISSOCIATED GLUCOCORTICOID RECEPTOR MODULATORS DECREASE THE COMPLEXITY IN BIOLOGICAL OUTCOME?

During the late nineties, a number of findings resulted in the transactivation versus transrepression hypothesis. It appeared evident at the time that we would be able to avoid particular steroid-induced side effects when finding ways to selectively trigger the GR-dependent transrepression pathway (which inhibits pro-inflammatory transcription factors NF-κB and AP-1, for example) without sparking the GRE-driven transactivation pathway. Especially the diabetogenic side effects that relied on transactivation of key pathway regulatory genes involved in the gluconeogenesis pathway (e.g. glucose-6-phosphatase and phosphoenol pyruvate carboxykinase), and that thus depend on a functional GRE in these target gene promoters, would be readily 'avoidable'. Some pieces of evidence in support of this hypothesis were as follows. First of all, upon treating cells with cycloheximide, a protein synthesis inhibitor, it was found that the repression fold of TNF-induced cytokine expression, e.g. IL-6, by glucocorticoids remained unhampered (60). This finding indicated that novel protein synthesis is not a direct need in the GR-mediated mechanism targeting cytokines. Secondly, the GR*dim* mouse model, a knock-in transgenic mouse model in which the GR dimerization abilities were compromised due to a GR A458T point mutation in the DBD, demonstrated that a hampered dimerization, subsequently also affecting DNA binding, was still compatible with GR-mediated cytokine transrepression mechanisms (61). These and other findings along the same lines (reviewed in (14)) were the start sign for many big pharma to embark on a quest to develop improved steroids, i.e. so-called dissociated glucocorticoids and, in a second wave, mostly non-steroidal selective GR modulators which favour transrepression over transactivation effects in an attempt to ultimately reduce the number and severity of GR targeting therapy-induced side effects (20,62,63). The first steroidal structures abiding to this sharply differentiating profile *in vitro* proved to be unsuccessful *in vivo* (64). Moreover, it was also found that the GR*dim* mutant could still allow GR-mediated transactivation on specific promoters (16,65). Hence, the GR*dim* mouse did not appear to be the strict transrepression-transactivation differentiation tool as first anticipated and would thus still be able to support certain specific GR-mediated transcriptional activation events. So far, the fact remains that it has not been easy to strictly dissociate anti-inflammatory effects from adverse effects based on the dissociated steroid paradigm, and momentarily the field seems heavily divided in believers and non-believers of

the transactivation versus transrepression hypothesis (50,66,67).

Well studied by our own research team, the one example of a selective GR modulator that has so far lived up to expectations is Compound A (CpdA) (68). In contrast to other GR modulators, CpdA, a derivative of a Namibian desert shrub isolate, was not engineered or did not emerge as a hit following natural compound library screens but rather found in a serendipitous manner (69). Collaborative work with the team of Louw, led to the characterization of CpdA as an NF-κB-targeting anti-inflammatory compound, via GR (68). Various *in vivo* inflammatory mouse models have demonstrated that CpdA's anti-inflammatory effects (70–73) can be combined with a reduction of the diabetogenic and hyperinsulinemic side effect profile (74), and also with a preservation of bone integrity (75). Unfortunately, stability issues of CpdA, which can be perfectly kept under tight control in a lab environment, resulted in a dead-end street for CpdA from a druggable perspective.

Both non-believers and believers of the transactivation vs transrepression hypothesis have accepted since a long time now that the assumption that *all* beneficial effects could be ascribed to transrepression, and *all* devastating effects should be attributed to transactivation, is just too simplistic and unnuanced. It is even way too naïve and almost an offense to nature to try to divide GR's molecular mechanisms in merely these two categories, since many more mechanisms have been described (49,76). As often, the truth will lie in the middle. For cases subject to GR resistance and particular inflammatory models (e.g. antigen-induced arthritis or LPS-induced septic shock) (77,78), an enhancement of the transactivation properties of GR may even be desirable (50,67). More studies are necessary to further sculpt and support this working hypothesis. For other cases and particular inflammatory models, e.g. PMA-induced skin inflammation (79), a more sustained and solid transrepression may be a more preferable end goal. In addition, trying to achieve tissue-selectivity, such as recently has been demonstrated for the estrogen receptor (80), is an interesting and important concept to kindle and investigate more, also for GR.

Nevertheless, this research area clearly needs more work and a further unravelment of the fundamental mechanisms of GR will undoubtedly be helpful to achieve this laudable goal. Strongly dissociating GR modulators fulfill an important role herein, since they have the power to reveal an even broader plethora of differential mechanisms that lie within GR's portfolio.

CONCLUSIONS

To be able to understand how glucocorticoids work, an integrated vision that assembles the input of all possible regulators is needed. The occurrence of steroid resistance is the illustration of a consequence of combined complexities that are gathered in an applied clinical setting. There are so many different steps

in the GR pathway that could be altered, resulting in an ultimate steroid resistance, yet arising from different molecular phenomena. For example, if one looks at the numerous factors that have been described to contribute to glucocorticoid resistance, a long list of players -which might well be non-exclusive contributors- emerges. This list contains, among others, altered GR isoform levels, GR post-translational modifications (PTMs) (25,81,82), miRNA's modifying the GC response (9,83) and altered levels of pro- and anti-apoptotic proteins (84). Mechanisms further downstream of GR are also implicated in GC resistance; it has been proven that various kinase pathways (e.g. ERK, JNK and mTOR) oppose GC-induced apoptosis, while others (e.g. p38 and PKA) promote it (85,86). Importantly, it has also been shown that although a decrease in GR levels can contribute to GC resistance (6,70), it is apparently not a prerequisite for GC resistance to occur in hematological malignancies (84,86). Bearing this complexity in mind, it is clear that a practical solution for steroid resistance is not readily in sight. The same conclusion can be drawn for other examples of the GR regulation and signaling complicatedness, such as the numerous side-effects provoked by the receptor.

It may seem that the more we come to know about the (complex) action mechanisms of GR, the further away we drift from the belief that one day we'll hear the pieces of the puzzle fall into place with a satisfying click, when it comes to getting rid of the side effects and upholding therapeutic potential. However, the ongoing joint effort of many researchers in the field should continue for the sake of the many sufferers from diseases for which the GR is a most welcome positive target. The number of patients in need of affordable immunosuppressive agents is increasing, and the usage of steroids as one of the eldest drugs on the market, is, despite the side effects and occurrence of resistance, not decreasing but on the contrary, steadily rising.

ACKNOWLEDGEMENTS

Sofie J. Desmet, is a predoctoral researcher at the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (FWO). Ilse Beck is a postdoctoral researcher at the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen. DC and JD are predoctoral researchers at Vlaams Instituut voor de Bevordering van het Wetenschappelijk-Technologisch onderzoek in de Inductrie (IWT).

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