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Review Article

Modulation of the Glucocorticoid Receptor Activity by Post-Translational Modifications

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Abstract. Glucocorticoids (GCs) regulate numerous physiologic processes in order to maintain homeostasis. Most of their actions are mediated by an intracellular GC receptor (GR). The dysregulation of the GR function has been associated with different pathologies such as stress-related disorders and inflammatory and autoimmune diseases. The final outcome of GC actions is regulated at multiple levels and has been extensively reported. Nowadays, novel insights into the modulation of the GR activity arise from the study of the multiprotein chaperone/cochaperone machinery, the nuclear receptor cofactors (coactivators and corepressors), and chromatin regulation and their concomitant impact on GR-mediated gene transcription. Nevertheless, the complexity of GR-mediated gene regulation cannot be explained by a finite number of chaperones and cofactors. A further level in the regulation of GR activity is achieved by posttranslational modifications (PTMs) in response to external stimuli. PTMs can regulate protein stability, structure, function, activity, intracellular localization, and interaction with other proteins during cellular processes. Therefore, dynamic regulation of the molecular properties of these proteins by PTMs allows for further understanding the complexity of GR-dependent gene expression and its impact on GR-mediated pathophysiological processes.

Keywords: glucocorticoid receptor, posttranslational modifications, chaperones, cofactors

1. Introduction

Activation of the hypothalamic-pituitary-adrenal (HPA) axis after exposure to a stressor is part of an adaptive response that enables an organism to respond appropriately to changes in the environment. Under control of the HPA axis, the adrenal cortex releases glucocorticoids (GCs) [1] in order to facilitate processes aimed at adapting to the stressor. GCs will trigger different responses regulating a variety of biological functions [2]. After the coordinated regulation of immune, endocrine, and neurological responses, GCs inhibit their own synthesis and thereby restore homeostasis. Due to its wide range of functions, the imbalance in the levels of GCs inevitably leads to a broad range of pathophysiological effects. In the brain, the dysregulation of GCs activity is associated with hippocampal degeneration and memory impairments [3], with higher risk for psychosis, as well as stress-related disorders [4, 5]. GCs are the most potent anti-inflammatory and immunosuppressive drugs currently in

clinical use. Their immunosuppressive actions are mediated by the downregulation of numerous inflammatory genes such as cytokines, chemokines, adhesion molecules, and enzymes [6, 7].

The majority of actions exerted by GCs occur by their binding to the GC receptor (GR) [8–10], which regulates the expression of GC-responsive genes positively or negatively. GR-mediated promoter activation can rely on DNA binding of a homodimeric GR on a GC response element (GRE) in the promoter (simple GRE), on a coordinated DNA binding of a GR/Transcription Factor (TF) complex onto a so-called composite GRE or on GR/TF tethering mechanism [11]. The latter two mechanisms can also form the basis for GR-mediated promoter repression [11]. Additionally, GR-regulated transcriptional repression can be exerted via DNA binding of monomeric GR directly onto a so-called negative GRE (nGRE) [12–14]. Many of the anti-inflammatory effects of GCs are mediated by repression of proinflammatory and immune-related TFs such as NF-κB and AP1, among others [15–23]. The endocrine and metabolic effects of GCs are mostly mediated via GRE, onto which GR can directly bind as a homodimer and drive gene expression [24–28]. However, a very recent work suggests that, contrary to dogma, there is no clear correlation between the GR monomeric/dimeric state and the mechanistic pathway the receptor will follow upon ligand binding [29]. This discovery presents supporting evidence towards the increasing view of the complexity of GC action.

In the absence of hormone, the receptor is present in the cytoplasm as a complex which contains chaperone, immunophilins, as well as other proteins which are implicated in regulating different aspects of GR activity [30] as will be discussed later. Upon ligand binding, the receptor translocates into the nucleus where it regulates the transcription of target genes. GR interacts with specific cofactors to implement a variety of gene promoter effects. However, this simplified signaling cascade does not unveil the extreme complexity of gene-, cell-, and tissue-specific activity of GCs [9, 31]. Diversity in GR signaling comes from the actions of different Diversity in GR signaling comes from the actions of different GREs and multiple receptor isoforms generated by alternative splicing and translation initiation [32–34]. GR activity and function are further modulated by posttranslational modifications (PTMs), which modify GR activity, and also indirectly by modulating the activity of GR-interacting proteins, such as the chaperone heterocomplexes and cofactors, expanding the diversity of GC responses [35]. The imbalance of GR activity has been extensively associated with inflammatory, autoimmune, and stress-related disorders. Understanding the influence of PTMs on the molecular mechanisms involved in GR signaling is thus of utmost importance in the search for therapeutic strategies aimed at modulating GR responses under pathophysiological circumstances. The current review aims to give an overview of the progress in our understanding of how GR-mediated activity is modulated by PTMs and how this contributes to the increasing diversity in GCs signaling pathways, focusing on the neuroendocrine and immune context in health and disease.

2. Glucocorticoid Receptor and Molecular Chaperones

GR activity is regulated by a dynamic chaperone and cochaperone multiprotein complex (Figure 1). The GR chaperone complex has an important function in assisting the proper protein folding of GR but also supports GR stability and ligand binding, facilitating its translocation into the nucleus and modulating GR-mediated transcriptional activation or repression of target genes [36]. This chaperone complex includes heat-shock proteins, such as Hsp90 and Hsp70, and immunophilins, such as the FK506-binding protein (FKBP) 51 and FKBP52 [37]. When binding to its ligand, GR undergoes a conformational change that promotes its translocation to the nucleus and the recruitment of regulatory cofactor complexes finally impacting gene transcription [38]. Interestingly, instead of being kept inactive in the cytoplasm of the cell, a rapid nucleocytoplasmic shuttling of the receptor underlies its localization [39] (Figure 1). Against previous beliefs, it is now accepted that GR does not dissociate from its chaperone complex when binding to its ligand but instead remains associated within this complex to translocate to the nucleus. GR nuclear translocation is controlled by the Hsp90 machinery, specifically by the recruitment of immunophilin FKBP52 to the GR–Hsp90 complex (Figure 1). The integrity of the chaperone complex seems to be critical for GR nuclear translocation [40, 41]. Considering the key regulatory function of these proteins, their regulation by PTMs, as will be later discussed, represents a novel level of modulating GR activity and therefore might become interesting therapeutic targets for the treatment of many associated diseases.

While Hsp70 is the molecular chaperone that is essential for the folding of nascent chains, it is Hsp90 which regulates the final maturation of GR by helping it to achieve a hormone-dependent activation state. The relevance of Hsp90 on GR activity has been extensively documented [37, 42–44]. Apart from ensuring ligand accessibility to the ligand-binding pocket, Hsp90 seems to enable hormones and coregulators to act as allosteric effectors, which forms the basis for gene- and cell-specific responses of GR to ligands [45].

In particular, pharmacological manipulation of Hsp90 function has become an important tool to shed light upon the importance of Hsp90 in regulating immune and neuroendocrine responses in a GR-dependent manner [46–50]. Inhibition of Hsp90 was found to interfere with the anti-inflammatory actions of GR [46, 47], apparently by attenuating GR inhibition of proinflammatory TFs NF-κB and AP1 [48]. Moreover, inhibition of Hsp90 chaperoning function in neuroblastoma cells leads to reduced GR transactivation by interfering with GR-Hsp90 association, followed

by proteasome-dependent degradation of the receptor [49]. It also impairs GR retrograde movement along neurites while inducing GR degradation by the proteasome as well [50]. In addition, the alteration of GR-Hsp90 interaction impacts on stress-related behavior *in vivo* due to reduced nuclear translocation and altered GR function during stress response [51–53]. As will be further discussed in this review, PTMs that target Hsp90 regulate the chaperone activity and further impact on GR activity.

Other components of the GR-Hsp90 heterocomplex play critical roles in regulating GR function. It was reported that immunophilin composition of the GR chaperone complex can modulate GR translocation, since FKBP51 inhibits GR nuclear transport while FKBP52 binding to dynein appears to be responsible for FKBP52-mediated enhancement of GR nuclear translocation [54–56] (Figure 1). Therefore, FKBP52 is regarded as a positive regulator of GR transcriptional activity [57] and FKBP51 as a negative regulator [58]. Accordingly, its overexpression prevents the positive regulation by FKBP52 because of the competition of FKBP51 for the same binding site on Hsp90 [57]. Interestingly, FKBP51 gene (*FKBP5*) mRNA and protein expression are induced by GR activation via intronic hormone response elements [59], suggesting the existence of an ultrashort negative feedback loop regulating GR activation. The GR-enhanced expression of FKBP51 in turn moves the equilibrium back towards the FKBP51-containing complexes, resulting in attenuation of GCs actions.

Adrenal secretion of GCs is one of the major mechanisms by which human responds to stress. Therefore, alterations in both FKBP51 and FKBP52 have been implicated in impaired GR signaling and stress-related disorders associated with HPA axis dysfunction [60, 61] such as depression and bipolar disorders [62]. This appears to be at least in part due to impaired efficiency of the negative feedback regulation by cortisol-loaded GR in the HPA axis. Single nucleotide polymorphisms (SNPs) in the *FKBP51* gene have been associated with increased expression of the cochaperone protein, which in turn may link to differences in GR activity and contribute to GCs resistance [63]. This deregulated stress response might be a risk factor for stress-related psychiatric disorders. These same alleles are overrepresented in individuals with major depression, bipolar disorder, and posttraumatic stress disorder and are also associated with faster response to antidepressant treatment [63]. Therefore, FKBP5 has been proposed as an interesting therapeutic target for the prevention and treatment of stress-related psychiatric disorders [63]. FKBP51 has also been associated with immune-related diseases and inflammation. Its role in these pathologies is apparently mediated not only by its cochaperone function but also by its ability to modulate NF-κB activity and its dependent gene expression [61]. On the other hand, FKBP52 has also been proposed as a therapeutic target based on results obtained in *in vivo* experiments with knockout mice [64, 65]. In particular, heterozygous FKBP52 knockout mice were found to display an altered phenotype regarding behavioral, neurogenesis, and neuroendocrine parameters under basal and chronic stress conditions. Alteration in these parameters is most likely due to reduced GR sensitivity of the HPA axis [65], highlighting the importance of FKBP52 regulation of GR activity. Taking into consideration that cochaperones, such as FKBP52, are targeted by PTMs, the complexity of GR regulation becomes of striking importance.

3. Glucocorticoid Receptor and Coregulators

Activation or repression of target genes is achieved by GR recruitment of coregulators that serve as coactivators or corepressors to responsive regulatory regions [66, 67]. GR agonists exert both GR-mediated transactivation and transrepression in a promoter and context specific fashion [68–70]. Targeting these proteins by PTMs, as will be exemplified later in this review, further regulates these processes (Figure 2).

To initiate transcription, GR uses its transcriptional activation domains, AF-1 and AF-2, as surfaces to interact with nuclear receptor coactivators and chromatin-remodeling complexes. Coactivators include a wide range of proteins that enhance nuclear receptor-dependent transcription through interaction with the ligand-bound receptor. They generally mediate interaction between nuclear receptors and the general transcription machinery. In addition, most of the coactivators also display enzymatic activities that contribute to their function in promoting transcription, such as histone acetyl-transferase (HAT) and histone methyl-transferase, supporting the key role of PTMs in regulating GR activity (Figure 1). They mediate chromatin remodeling and facilitate the association of RNA polymerase II (RNA Pol II) complex with the general transcription machinery at the promoter of the target gene [71]. The N-terminal domain (NTD) AF-1 contributes to the interaction of GR with cofactors, chromatin-remodeling enzymes, RNA Pol II, the TAT-binding protein, and TBP-associated proteins (TAFIIs). However, the C-terminal domain (CTD) which harbors the ligand-binding domain (LBD) of GR can also accommodate coactivator binding to the C-terminal AF-2 domain [72]. These GR-bound multisubunit coregulator complexes can consist of p300 or CBP, p/CAF, steroid receptor coactivators SRC1, SRC2, and/or SRC3, all of which possess HAT activities, and also PGC-1a, which can recruit HAT activity-containing cofactors, such as SRC-1, p300, or DRIP/TRAP. The GR-bound enhanceosome of promoters governed by GREs could also contain the ATP-dependent chromatin-remodeling complex SWI/SNF and/or elements of the DRIP/TRAP complex [73].

Recruitment of corepressors by unliganded or antagonist-bound nuclear receptors partly accounts for inhibition of gene expression. Two major corepressors identified to interact with GR are nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptor (SMTR)

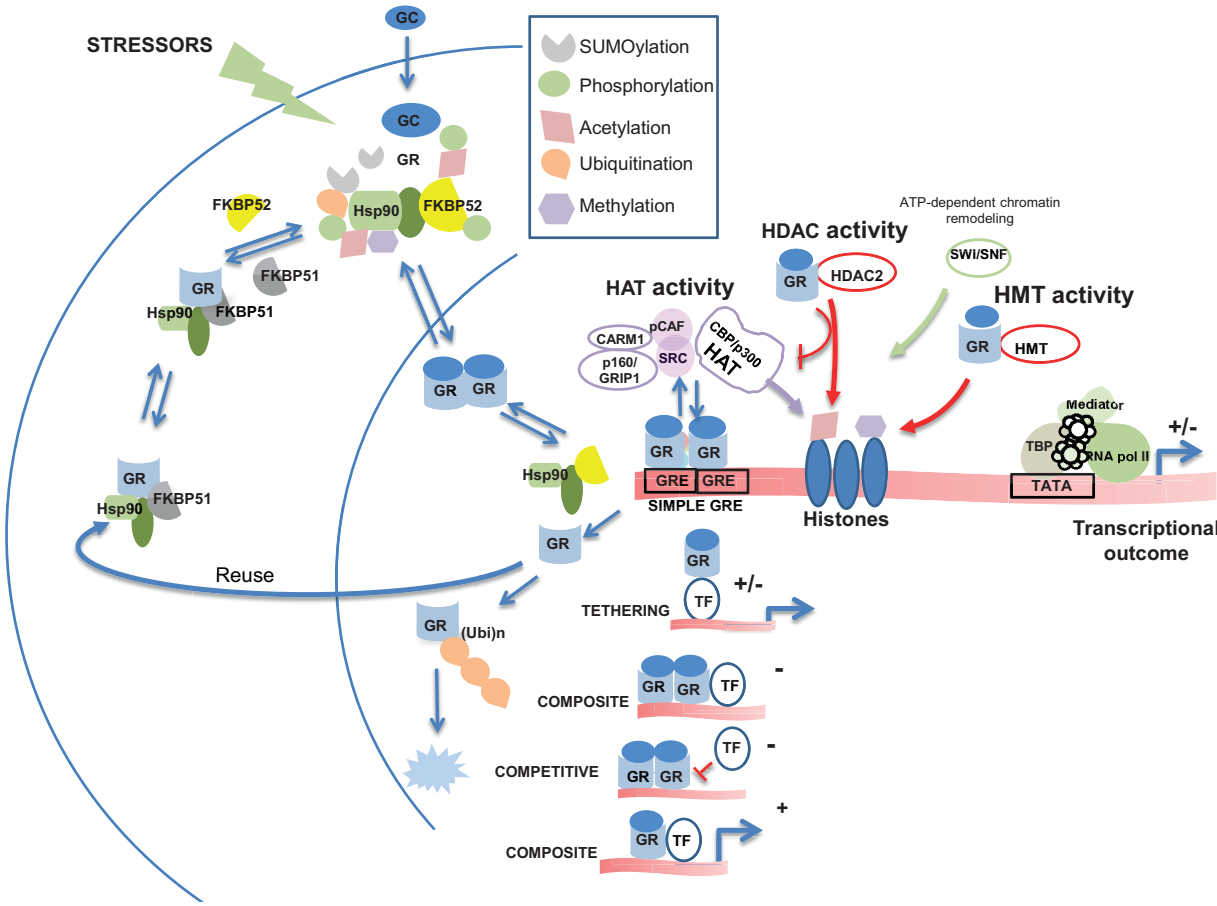

Figure 1: **GR activity is regulated by the chaperone/cochaperone complex and also by coactivators/corepressors which in turn are targets of posttranslational modifications.** The GR is associated with chaperones (e.g. Hsp90) and cochaperones (e.g. FKBP51 and FKBP52), which are implicated in regulating the function, folding and trafficking of the GR. Upon ligand binding, FKBP51 is exchanged for FKBP52 and translocates to the nucleus. Several PTMs directly target GR and also GR heterocomplex further regulating its activity. GR-mediated promoter activation relies on GR DNA binding on simple GREs, on a coordinated binding of a GR/TF complex onto composite GRE or on GR/TF tethering. The latter two mechanisms are also implicated in GR-mediated repression. GR uses chaperone/cochaperone complexes containing Hsp90 to facilitate dynamic interactions with target sites. Hormone release from GR and GR release from chromatin might require complexes with Hsp90.GR may be ubiquitinated and degraded by the proteasome or reused. Coactivator and corepressor are required for GR-mediated transcriptional regulation. Most recruited coactivators display enzymatic activities, such as histone acetyl-transferase (HAT), histone methyl-transferase (HMT), and ATP-dependent chromatin remodeling. They mediate chromatin modification and facilitate the association of RNA polymerase II complex with the general transcription machinery. Corepressors include ATP-dependent chromatin remodeling complexes, basal corepressors, and subcomplexes that may contain histone deacetylase (HDAC) activity and specific corepressors. The GR can bind to coactivators to inhibit HAT activity directly and recruite HDAC2, which reverses histone acetylation leading to suppression of TF-activated inflammatory genes. Therefore, HATs, HDACs, and HMTs support the key role of PTMs in indirectly regulating GR activity.

[74–76]. The sites of interaction in steroid and nuclear receptors for both corepressors and coactivators have been identified to be in the ligand-binding domain (LBD); in fact, the two sites appear to overlap [77]. PTMs that target this site affect GR transcriptional outcomes as will be further discussed [78].

An interesting example arises from glucocorticoid receptor interacting protein (GRIP) 1, which is recruited by GR upon ligand-binding. GRIP1 belongs to the p160/steroid receptor coactivator (SRC) family of coregulators [73]. GRIP1 displays not only coactivator activity, but also corepressor activity when they are recruited to GR tethered AP-1 or NF-κB target sites [79]. GRIP1 activity is context dependent but is also influenced by epigenetic regulators, context, and other unrecognized regulatory determinants [80]. It has been described that GRIP interacts with the HMT, Suv4-20h1. Suv4-20h1 is known exclusively as a factor involved in constitutive heterochromatin maintenance, but, when associated to GRIP1, it actively participates in hormone-dependent transcriptional regulation affecting

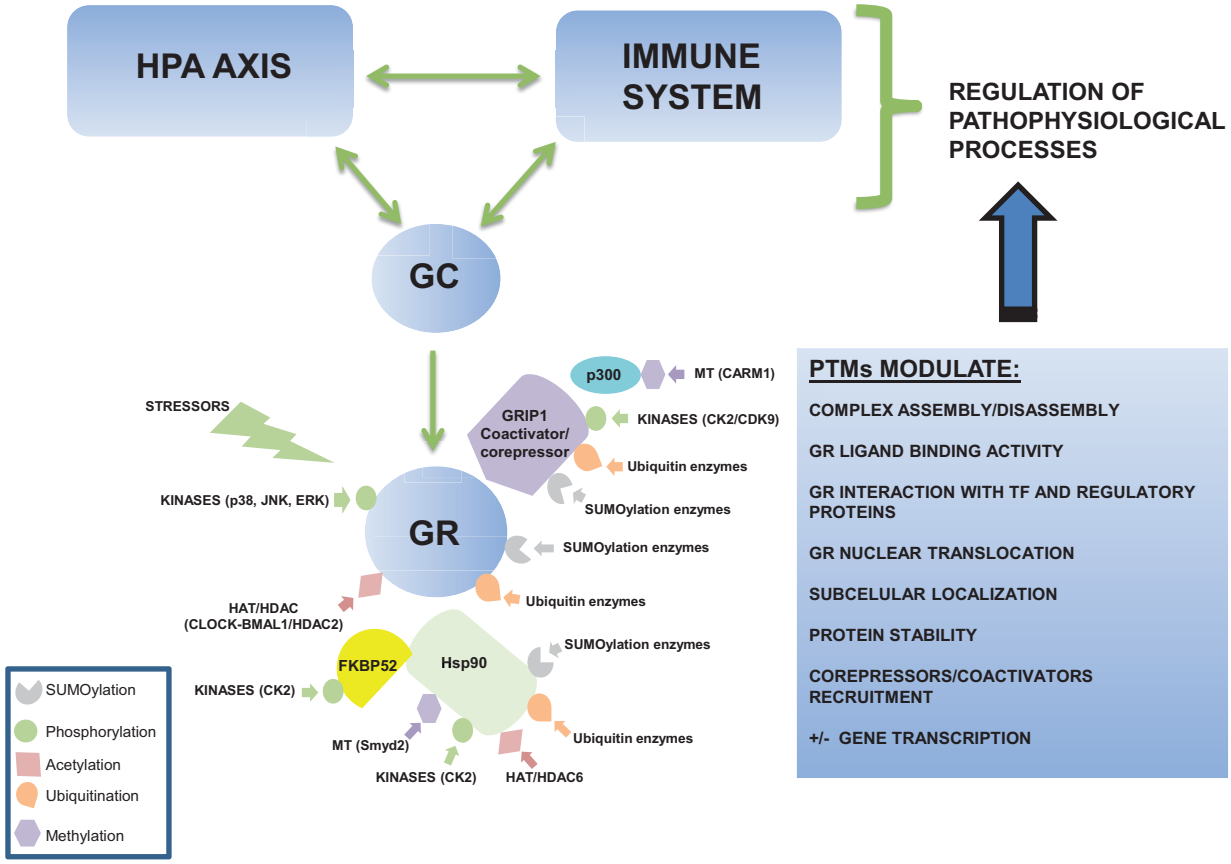

Figure 2: **Posttranslational modifications play a crucial role in the regulation of GR activity which impacts on the neuroendocrine and immune regulatory pathways.** GCs are the downstream effectors of the HPA axis response. They play a key role in the communication between the neuroendocrine and immune systems to ensure homeostasis. GCs impact on both HPA-mediated stress responses and immune responses. GCs effects are mainly mediated through binding to the GR. A series of mechanisms regulate GR activity to ensure GCs specificity. These mechanisms include modulation of GR by posttranslational modifications (PTMs). Different cellular stressors and also hormone binding can modulate PTMs of target proteins. PTMs do not only target GR but also key molecules involved in the regulation of GR activity such as the cochaperone/chaperone heterocomplex and GR coactivators and corepressors. PTMs regulate protein properties including stability, structure, function, activity, intracellular localization, and interaction with other proteins during cellular processes determining the final outcome. This review focuses on PTMs that target GR and GR modulators such as Hsp90, FKBP52, and GRIP1, fine-tuning GR transcriptional outcome, thus adding complexity and specificity to GCs action. GR and Hsp90 activity is modified by SUMOylation, ubiquitination, acetylation, and phosphorylation. Hsp90 is further regulated by methylation by methylatransferases (MT). GRIP1 is targeted by phosphorylation, ubiquitination, and sumoylation. Finally, FKBP52 by phosphorylation and p300 by methylation.

GR target gene expression in a promoter- and cell type-specific manner [81]. A recent study on GR regulation of LPS-stimulated macrophages gene expression showed that GRIP1 is equally recruited to both up- and down-regulated genes [80]. Mechanisms switching GRIP1 from coactivator to corepressor or vice versa depending on the context remain yet to be elucidated but may also be influenced by different PTMs as will be further discussed.

Other corepressors that are recruited by GR are histone deacetylases (HDACs) (Figure 1). Histone posttranslational modification by acetylation is mediated by transcriptional coactivators, which have intrinsic HAT activity, whereas repression is induced by HDACs, which reverse this PTM, allowing for repackaging of the nucleosomes, and, therefore, is generally related with transcriptional repression [82]. Therefore acetylation/deacetylation by these enzymes indirectly modulates GR transcriptional outcome. Both SMRT and N-CoR interact directly with multiple HDACs [83, 84] and may associate with HDACs 1 and 2 via the Sin3 repressor [85]. The current view of SMRT and N-CoR function holds that these corepressors not only deliver HDACs to target genes but also serve as critical cofactors in the formation of an active HDAC enzyme [86]. Recruitment of HDACs to GR negatively regulated genes contributes to inhibiting their expression. In this regard, HDAC2 has

been shown to reduce histone acetylation at the activated inflammatory gene promoter complex, thereby effectively suppressing activated inflammatory gene transcription [87–89]. As will be further discussed, HDAC also directly targets GR and Hsp90 regulating its activity [88, 90]. In the context of psychiatric diseases, selective HDAC 1/2 inhibition modulates chromatin and gene expression in brain and alters mouse behavior in mood-related tests [91]. Also, HDAC1 has been shown to participate in the down-regulation of corticotropin-releasing hormone gene (CRH) expression by GCs [92]. Collectively, these data point to a role for HDAC1 in the physiopathological regulation of CRH.

Similarly to GRIP1 dual activity in the regulation of gene transcription, HDACs have also been associated with GR-dependent transcriptional activation. HDAC1 was shown to be required for ligand-induced GR-dependent MMTV promoter activation [93]. HDAC2 was also found to be critical for GR-mediated transactivation, since knockdown of HDAC1 or HDAC2 separately decreased MMTV transcription [94]. HDAC involvement in GR-mediated transcription seems to have genome-wide effects [95] and the final outcome should be carefully considered. These enzymes do not only target GR and its coregulators as discussed but also may themselves be target of other PTMs that may influence the final transcriptional outcome.

4. Glucocorticoid Receptor and Posttranslational Modifications

The existence of an enormous number of receptor variants, having differential characteristics in expression, localization, and transcriptional activity, comprises a tissue- or cell-specific GR population providing an important mechanism for regulation of GR action [9, 96–98]. However, PTMs represent an important mechanism in the regulation of GR signaling upon ligand binding. These PTMs target not only GR and GR variants but also key molecules involved in the regulation of GR activity. PTMs allow for the regulation of protein properties including stability, structure, function, activity, intracellular localization, and interaction with other proteins during cellular processes determining their final outcome. We will focus in this matter showing key examples on how PTMs that target GR and its modulators (e.g., Hsp90, FKBP52, and GRIP1) fine-tune GR transcriptional outcomes adding complexity and specificity to GCs action.

5. Phosphorylation

Phosphorylation is an important PTM for the regulation of protein function. The GR is a phosphoprotein, and phosphorylation modulates its activity (Figure 2). GR phosphorylation occurs in a hormone-dependent manner, at serine/threonine residues located within the DNA-binding domain [99]. Phosphorylation of GR has been shown to be critical for its activation. Many target phosphorylation residues have been characterized up to date, most of which are located in the AF-1 domain of its NTD [100]. Phosphorylation at each residue has a specific effect on GR activity that can be either positive or negative [101]. For instance, p38 mitogen-activated kinase- (MAPK-) dependent GR phosphorylation at serine 211 was shown to be critical for the induction of AF1-domain conformational change that subsequently facilitated interaction with coregulators and activation of transcription [102]. It was demonstrated that differentially phosphorylated GR species show specific intracellular sublocalization [103]. Intriguingly, specific GR phosphorylated forms were differentially recruited to promoters of target genes and selectively regulated their expression. In addition, phosphorylation status of individual residues seems to have different impact depending on the target gene under analysis [104, 105]. Recently, it was described that GR phosphorylation occurs not only in a hormone-dependent manner but also, previous to hormone binding, as a consequence of cellular stress, therefore regulating GR response upon ligand stimulation. This newly described mechanism suggests that cellular history prior to GCs signaling, measured by phosphorylation of the GR, has an impact on the regulation of its target genes [106]. In addition, GR protein stability has been shown to be dependent on its phosphorylation state, since phosphorylation mutants displayed increased protein stability and decreased sensitivity to ligand-induced reduction in protein levels [107].

Many different stress conditions are related to the modulation of PTMs on target proteins. Cellular stresses such as starvation or oxidative stress induce p38 MAPK-dependent phosphorylation of Ser134. This phosphorylation mediates GR interaction with 14-3-3 ζ, a protein associated with oxidative stress, GR binding to selective gene promoters, and alters the GR-mediated target gene profile [106]. Aberrant GR phosphorylation has been proposed to play a role in disease. For example, some GC-resistant asthma patients become responsive when p38 MAPK inhibitors are given with reduced Ser226 phosphorylation as one of the results of kinase inhibition [108]. In women, the ratio of nuclear phospho-Ser211/phospho-Ser226 measured in peripheral blood mononucleocytes is inversely correlated with depression [109]. In this regard, phosphorylation of GR was shown to be altered due to stress and antidepressant treatment, rendering GR phosphorylation a putative target for antidepressant actions. In the chronic mild stress model of depression, alterations of GR trafficking and transcription in the hippocampus and in the prefrontal cortex were suggested to be sustained by changes in receptor phosphorylation. In this same work, antidepressant treatment normalized these alterations [110]. In line with these results, it was demonstrated that antidepressant treatment increases human hippocampal neurogenesis via a GR-dependent mechanism that requires PKA signaling, GR phosphorylation, and activation

of a specific set of genes [111], rendering GR phosphorylation a putative target for antidepressant actions.

Interestingly, during inflammation, stimulation through proinflammatory signals culminates in the activation of AP-1 and NF-κB, together with other relevant inflammatory TFs, which in turn induce the expression of proinflammatory genes. Activation of such inflammatory pathways involves the participation of kinases. Remarkably, these same kinases (JNK, p38, and extracellular-signal regulated kinases (ERK)) are involved in modulation of GR activity through phosphorylation. At the same time, GR regulates activation of these pathways by modulating the activity of the kinases, thus contributing to the complexity of the landscape [101] (Figure 2). Moreover, chaperones complexes and coregulators are also targeted by phosphorylation, which alters their functions and thus impacts on GR activity [112–114]. The immunophilin FKBP52 binds to Hsp90 via its TPR domain and is important for chaperoning of steroid hormone receptors. Casein Kinase II (CK2) phosphorylates FKBP52 on Thr-143 both *in vitro* and *in vivo* (Figure 2). Phosphorylation of this residue does not affect FK506 binding to FKBP52, but phosphorylated FKBP52 does not interact with Hsp90. These findings suggest that phosphorylation of FKBP52 plays a role in modulating steroid hormone receptor-mediated signal transduction [115, 116]. Hsp90 is a phosphoprotein and its steady-state phosphorylation level is influenced by different cellular environments in a species-specific manner [117] (Figure 2). A number of serine, threonine, and tyrosine phosphorylation sites have been identified in Hsp90. Hsp90 phosphorylation can affect its ability to chaperone client proteins [118] and therefore may impact on GR activity [119]. As previously mentioned, GRIP1 p160 family member functions as coactivators for GR. Unlike other p160s, GRIP1 also potentiates GR-mediated repression of AP1 and NF-κB targets and, surprisingly, transcriptional activation by interferon regulatory factors. What enables GRIP1 activating or repressing properties is unknown. It has recently been demonstrated that GRIP1 undergoes GC-induced, GR interaction-dependent phosphorylation by two putative GRIP1 kinases, CKII and cyclin-dependent kinase 9 (CDK9), and also that GRIP1 phosphorylation potentiates GR-mediated activation of transcription [120] (Figure 2). These findings suggest that GR actively imparts modifications that dictate GRIP1 function, adding a layer of specificity to GR transcriptional control.

6. Acetylation

Like other nuclear receptors belonging to the same superfamily, the GR is acetylated in lysine residues within its DNA binding domain (Figure 2). The acetylated GR is deacetylated by histone deacetylase 2 (HDAC2) and this deacetylation is necessary for the GR to be able to inhibit NF-κB activation of inflammatory genes [88]. By directly targeting acetylated GR, HDAC2 potentiates the inhibitory effect of GCs [88] (Figure 2). Moreover, the deacetylation of GR by HDAC2 seems critical for the interaction between p65 and the receptor [88]. In particular, HDAC2 levels were found to be critical for GCs insensitive response in patients suffering from a chronic inflammatory disease and chronic obstructive pulmonary disease (COPD). Primary alveolar macrophages from these patients presented low HDAC2 protein levels. Overexpression of HDAC2 in GC-insensitive macrophages restored GCs sensitivity [88], pointing to a key role for HDAC2 and GR acetylation in the regulation of inflammatory immune responses. Acetylation/deacetylation of GR was found to be relevant not only for transrepression but also for transactivation, since the HAT protein CLOCK and BMAL1 repressed GR transcriptional activity by acetylating GR target lysine residues [121] (Figure 2). As previously mentioned, together with GR, other proteins directly or indirectly regulating GR activity are modified by acetylation. In this context, the most relevant target of acetylation is Hsp90 (Figure 2). HDACs can also influence gene expression indirectly. For example, HDAC6 can affect GR function, by regulating Hsp90's acetylation, which subsequently influences GR nuclear translocation [90]. Deacetylation of Hsp90 by HDAC6 was found to be critical for GR complex maturation. In HDAC6-deficient cells, GR activity was compromised as evidenced by defective GR ligand binding, nuclear translocation, and transcriptional activation [90]. In line with these results, the lack of HDAC6 results in deregulation of GR-Hsp90 complex assembly/disassembly and thus GR activity [122]. Interestingly, a recent study on the relevance of Hsp90 acetylation and its impact on GR function in a murine model of traumatic stress showed that deacetylation of Hsp90 by HDAC6 modulates GR downstream signaling in the brain, with an effect on stress-related behaviors [53, 123]. Therefore, HDAC activity appears to be important not only for transrepression regarding immunosuppressive and anti-inflammatory actions of GCs but also at the neuroendocrine level [124]. On the other hand, it has been demonstrated that Hsp90 acetylation regulates its interaction with client proteins, including cochaperones such as p23 and FKBP52 [125].

7. Methylation

Even though much research has focused on GR expression regulated by DNA methylation at the transcriptional level, little is known on how protein methylation can alter GR transcriptional activity. Interestingly, protein methylation has been associated with modulation of nuclear receptors coregulators. It has been shown that certain coactivators, such as p300, are methylated in the C-terminal region by arginine methyltransferase (CARM1) (Figure 2) leading to inhibition of interactions between p300 and GRIP1 [126]. Therefore, methylation of GR coactivators can also modulate GR signaling. Remarkably, Hsp90 has also been identified as a methylation target (Figure 2) of the cytoplasmic lysine

methyltansferase Smyd2 [127, 128] though its impact on GR signaling has not been reported yet.

8. Ubiquitination

Ubiquitination is another important PTM that cells use to target specific proteins, via the covalent attachment of multiple ubiquitin molecules, to the proteasome for degradation. Ubiquitin is a highly conserved molecule universally distributed among eukaryotes. The molecule is first activated by E-1 activating enzymes, then transferred to E-2 conjugating enzymes, and subsequently passed on to E-3 ligases [129]. E-3 ligases recognize a wide range of target substrates by their conserved ubiquitination motifs and attach ubiquitin to the appropriate residues on the target proteins. Once tagged, the proteins are degraded by the proteasome. The GR is also a target for ubiquitination thus marking it for degradation by the proteasome [130] (Figures 1 and 2). The ubiquitin–proteasome mediated degradation pathway regulates the GCs signaling system by controlling the degradation rates of GR. Proteasome inhibition leads to decreased ligand-induced GR protein downregulation and enhanced GR transcriptional activity [131, 132]. In support of these findings, mutation of the ubiquitin-target lysine within the PEST motif—these motifs are associated to protein degradation by proteasome—mimics the effect of proteasome inhibition, rendering GR protein levels independent of ligand-induced degradation and enhancing GR transcriptional activity as well [131, 133]. In addition, proteasome inhibition alters GR nuclear trafficking together with GR binding to the nuclear matrix [132]. Interestingly, inhibition of proteasome activity affects GR-target gene expression not only by altering GR proteasomal degradation but also by modulating histone methylation and RNA Pol II association with chromatin. Trimethyl histone H3 lysine 4, widely correlated with an active chromatin status, was demonstrated to be enriched at the MMTV activated gene when proteasome activity is inhibited both under basal conditions and upon hormone treatment. Moreover, density of activated RNA polymerase II at this gene was also found to be increased when inhibiting proteasomal activity [134]. Therefore, a link between chromatin structure and proteasome activity at GR target genes arises as a plausible explanation beyond GR proteasome degradation [134, 135]. Proteasome components are also found at the MMTV promoter regulating rapid GR exchange at this site, pointing to the proteasome as a regulator of hormone sensing and fine-tuning of GR responses to variable conditions [136]. Also, an E3 ubiquitin ligase has been identified for GRα, and alterations in the expression of this enzyme modulate receptor levels and cellular responsiveness to GCs [137].

The GR protein is subjected to hormone-dependent down-regulation in most cells and tissues. However, conflicting results have been obtained from *in vitro* and *in vivo* studies in maturing and developing neurons regarding the effects of GCs-mediated regulation of GR protein levels [4, 138, 139]. In hippocampal neurons, chronic GCs exposure does not alter GR protein levels, probably due to an unappropriated maturation of proteasomal degradative or targeting activities [139]. Accordingly, overexpression of the E3 ubiquitin ligase CHIP (C-terminus of Hsc70-interacting protein) [137, 140] abolishes the steroid-binding activity and transactivation potential of the GR, even though it has little effect on its synthesis. Instead, CHIP induces ubiquitination of the GR and degradation through the proteasome. Therefore, these results suggest that relative abundance of an E3 ligase might confer differential GR sensitivity in the neuronal context. Interestingly, CHIP is a component of the Hsp90 heterocomplex [140] and also targets Hsp90 for proteasomal degradation [141–143], further regulating GR activity [140].

Together with GR, nuclear receptor coregulators are also subjected to regulation by the ubiquitin/proteasome pathway [144]. In particular, GRIP1 is ubiquitinated in a cAMP-dependent manner, suggesting GRIP1 ubiquitination as an additional mechanism for GR-mediated transcriptional regulation [145] (Figure 2). Interestingly, components of the ubiquitin/proteasome pathway have also been described to act as nuclear receptor coregulators themselves, thereby providing a new link between nuclear receptors and the proteasome/ubiquitin pathway [135]. As an example, Ube3a, an E3 ubiquitin ligase, was shown to enhance ligand-bound GR degradation and also to act as a GR transcriptional coactivator [135]. This ubiquitin ligase plays a critical role in Angelman syndrome, a neurodevelopmental disorder. GR-mediated signaling was impaired in the brain of ube3a maternal-deficient mice, a murine model of Angelman syndrome [146]. At the same time, these mice showed increased chronic stress and anxiety-like behavior, probably due to impaired GR signaling regulating the HPA axis. Also, Hsp90 is a ubiquitination target (Figure 2) and, as a consequence, Hsp90 chaperone function is inhibited [147, 148].

9. SUMOylation

Small ubiquitin-related modifier (SUMO) is an 11 kDa protein moiety that can be covalently ligated to lysine residues in a variety of target proteins. The protein is similar to ubiquitin in size and three-dimensional structure, yet the functional consequences of SUMOylation are distinct [149]. While ubiquitination largely leads to the proteasome-mediated target protein degradation, modifications by SUMO regulate more diverse biological effects including protein-protein interactions, subcellular localization, protein stability, and transcriptional capacity [150, 151]. SUMO conjugation has also been found to play an important role in modulating GR transcriptional activity [152, 153]. Covalent attachment of SUMO to GR takes place in the absence of ligand (Figure 2), but GR agonists increase SUMOylation of the receptor [154]. A quantitative proteomic analysis of the SUMOylation states of proteins in response to heat shock identified GR as a SUMO target [155], suggesting a role for GR SUMOylation

in the modulation of its transcriptional activity under cellular stress. GR contains three consensus SUMOylation sites. The first two are located in the NTD while the third one is located in the LBD. The NTD SUMOylation sites are part of the synergy control (SC) motif sequence [152] which consists of short regulatory sequences that limit the synergistic transactivation in a promoter-dependent manner. It is well established that SUMO modification of the two NTD SUMOylation sites in the GR is responsible for the functional effect of the SC motifs, thereby exerting a negative effect on GR transcriptional activity at multiple GREs without altering GR-mediated transcription at promoters containing only a single GRE [152]. SC motifs limit transcriptional synergy of multiple DNA-bound regulators at compound binding sites, so that disruption of these motifs increases their activity. Thereby, SC motifs do not affect intrinsic activity of TFs but rather modulate their ability to synergize at compound-binding elements [156]. Based on these results, it has been hypothesized that GR SUMOylation within its SC motif would affect recruitment of corepressors and therefore transcriptional regulation of downstream genes [156, 157]. In line with these results, SUMO modification of GR at the N-terminal sites influences transcriptional regulation depending on context promoter, since no effect of SUMO conjugation to GR could be detected at the MMTV promoter [154]. Interestingly, we have demonstrated that a RWD-containing SUMOylation enhancer, RSUME [158], is responsible for SUMO conjugation to the LBD of the GR under heat stress conditions and uncovers a positive role for SUMO in GR-mediated transcriptional regulation during stress adaptation [78]. SUMOylation at this residue may be critical for GRIP1 cofactor-mediated GR activity, since its point mutation diminishes GRIP1 coactivator activity while it does not disrupt GR-GRIP1 interaction [78].

A genome-wide analysis of GR SUMOylation impacts on gene expression revealed that both hormone up- and downregulated genes are affected by SUMO modification of the GR, and that genes differentially regulated are related to proliferation and apoptosis pathways [159].

SUMO modification of the GR is influenced by other PTMs, since it was demonstrated that c-Jun N-terminal kinase- (JNK-) dependent phosphorylation enhances GR SUMOylation to fine-tune GR transcriptional activity in a target gene-specific manner [160].

Since regulators of the SUMOylation pathway such as RSUME are induced under stress [78, 158, 161], they might contribute to fine-tuning the cellular response to GCs during stress adaptation. Thus, understanding these mechanisms might contribute to the establishment of potential targets to modulate physiological and therapeutic responses to GCs.

Coregulators are also subjected to SUMO modification, as exemplified by Hsp90 and GRIP1 (Figure 2). SUMOylation of Hsp90 has been reported previously [162–164]. Interestingly, SUMOylation of an N-terminal domain lysine conserved in both yeastand human Hsp90 facilitates both recruitment of the adenosine triphosphatase- (ATPase-) activating cochaperone Aha1 and the binding of Hsp90 inhibitors, suggesting that these drugs associate preferentially with Hsp90 proteins that are actively engaged in the chaperone cycle, providing a mechanism to explain the sensitivity of cancer cells to these drugs [165]. GRIP1 is subjected to SUMO-1 modification. Lysine residues 239, 731, and 788 of GRIP1 serve as principal attachment sites for SUMO-1. Lys-731 and Lys-788 are located in the nuclear receptor interaction domain (NID), and their substitution by arginines impairs the ability of GRIP1 to colocalize with androgen receptor in nuclei, modifying the ability of GRIP1 to function as a steroid receptor coactivator [166].

10. Concluding Remarks

Since GCs effects are mainly mediated by GR, the development of therapeutic strategies necessarily requires the understanding of the underpinning molecular mechanisms implicated in the regulation of GR biological activity. In this regard, PTMs are important modulators of GR activity. However, the relevance of these PTMs on GR activity should be carefully analyzed considering the cellular context. The occurrence of these PTMs contributes to the development of tissue-specific responses. Therefore, understanding how PTMs impact on GR activity (by directly targeting GR or indirectly by targeting its coregulators) is of crucial relevance. Particularly, since GCs play a critical role in the regulation and communication between the neuroendocrine and immune systems ensuring the homeostatic balance (Figure 2), the knowledge of this regulatory level is fundamental for the development of novel therapeutic approaches aimed at differentially modulating GR function in order to overcome pathologies in which these systems are involved.

Conflict of Interests

The authors declare that there is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

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