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Review

Molecular mechanisms of the glucocorticoid receptor in steroid therapy – lessons from transgenic mice

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Abstract

Glucocorticoids (GCs) are potent anti-inflammatory agents that are used to treat chronic inflammatory diseases, allergic conditions, and some cancers. However, their therapeutic effects are hampered by severe side effects, such as muscle weakness, insulin resistance, fat redistribution, and osteoporosis. GCs act on many cell types that express the GC receptor (GR) via several modes of action. One of them includes GR homodimers recognizing binding sequences in the DNA of gene promoters. Another mode involves the modulation of other DNA-bound transcription factors via dimer-independent mechanisms. To what extent these mechanisms contribute to GC-mediated effects is currently being elucidated from analyses of mice with conditional and function-selective mutations of the GR and is summarized in this review. Whether GR homodimerization or its monomer activity is decisive in the therapeutic effectiveness and associated side effects of GCs for the treatment of inflammatory conditions depends on the type of the pathological condition. Thus, the classic criterion for selective GR modulators, discrimination between GR dimer- and GR monomer-dependent protein-protein interaction, will not help in any condition to avoid side effects and maintain anti-inflammatory activity. Rather, novel criteria for selective GR modulators have to be defined that take into consideration the tissue-specific mechanisms of the GR to achieve optimized anti-inflammatory therapies with reduced side effects. In the case of avoiding osteoporosis as a side effect, a first example of such optimized compounds can be provided.

Keywords: conditional knockout mice; glucocorticoid receptor; inflammation; osteoporosis.

Introduction

Since the middle of the last century, glucocorticoids (GCs) have been widely used as anti-inflammatory agents for the treatment of allergic conditions, chronic inflammatory diseases, and some cancers in which the initial cause is unknown or difficult to combat (1). This is surprising because due to their undisputed severe side effects, GCs would not pass current regulations for drug approval in most countries. The side effects include type 2 diabetes, osteoporosis, muscle weakness, skin atrophy, and even depression upon long-term use (2).

Their receptors, in particular, the classic GC receptor (GR), act in a versatile manner from altering gene expression as transcription factors to interfering with signaling pathways in a non-genomic fashion. Therefore, research on the molecular mechanisms of GC action is of importance to shed light on physiological processes under GC control. This should generate rationales to achieve specific drug development with the goal of anti-inflammatory therapy with fewer side effects.

Here, we summarize the pivotal findings and recent progress regarding cell-type-specific GR mechanisms of action revealed by genetic alterations of the GR in mice.

Endogenous GCs

GCs are elevated in a diurnal rhythm and during acute stress responses (3). Their secretion is pulsatile and controlled by a feedback mechanism involving hormones of the hypothalamus and pituitary (hypothalamus-pituitary-adrenal axis) (4). The hypothalamus releases corticotropin-releasing hormone (CRH) triggered by the circadian rhythm, systemic inflammation reactions, or mental stress. Subsequently, CRH causes secretion of proopiomelanocortin (POMC) from the anterior pituitary, which in turn is proteolytically cleaved into adrenocorticotropic hormone, the latter acting on the adrenal gland cortex to enhance production of corticosteroids (4). High concentrations of GCs lead to a suppression of POMC and CRH in the pituitary and hypothalamus, respectively, resulting in a decreased GC release. In rodents, the main GC is corticosterone, whereas in humans, it is cortisol or hydrocortisone (5). Within cells, the restricting factor for the production of active cortisol is 11-β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which converts inactive cortisone to the active metabolite. Conversely, active cortisol can be modified by 11-β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) via conversion of the hydroxyl group to a keto group at position 11, which in turn leads to inactive cortisone (6).

One important function of GCs is to allow the mobilization of glucose for rapid energy supply to the brain and by supporting gluconeogenesis (7). In addition, decisive roles in behavior (8) and inflammation (9) have been uncovered. Two disease states demonstrate the involvement of GCs in the regulation of glucose and energy metabolism (10). Addison's disease

patients with chronic low GC levels experience stress sensitivity, lymphoid tissue hypertrophy, weight loss, and hypoglycemia (11). Cushing's disease patients with chronic GC excess display hyperglycemia, liver steatosis, hypertension, elevated cholesterol, immunodeficiency, and insulin resistance (10). Further studies have been performed with rodent models of impaired GC secretion by adrenalectomy, recapitulating the Addison phenotype (10) and demonstrating an early role of endogenous GCs in systemic inflammation (12, 13).

In addition, multiple aspects of the role of endogenous GCs during development and in adult physiology have been revealed after introducing loss-of-function mutations for the GR in mice and inactivating GC activity in mice overexpressing 11β-HSD2 or being devoid of 11β-HSD1. With these genetically modified mice, it could be shown that endogenous GCs are required for the differentiation of chromaffin cells, lung maturation during development (14, 15), and bone integrity (16–18).

Thus, it is not surprising that because of the multiple roles of endogenous GCs in various physiological processes, the presence of excessive GCs during anti-inflammatory therapy leads to multiple side effects. In the following section, we briefly summarize how GCs act at the molecular level. We then describe the contributions of different molecular mechanisms to GC actions that have been elucidated in animal models.

The GC receptor

Non-genomic effects

GCs bind to the GR, a member of the nuclear receptor family. Nuclear receptors are ligand-induced transcription factors and share a common homology and domain structure: an N-terminal transactivation domain (AF-1), followed by a DNA-binding domain (DBD), a subsequent hinge region and a C-terminally located ligand-binding domain (LBD) associated with a second transactivation domain (AF-2) (19) (Figure 1A).

Despite being considered a transcription factor, rapid non-genomic effects have been described. In particular, interference of the GR with components of signal transduction pathways, such as PI3K, JNK, and 14-3-3 proteins, has been reported (20–22). Recently, Lowenberg et al. (23) suggested a mechanism underlying non-genomic GC-induced immunosuppression in T cells by membrane-associated liganded GR disrupting the protein kinase Fyn complex and impairment of T-cell receptor (TCR) activity. GC effects may also be mediated by non-specific interactions with cellular membranes or by specific interactions with membrane-bound GR (24). However, a clear identification of these GR types is lacking and remains a challenge.

Regulation of gene expression by DNA binding of the GR

In the presence of hormones, the GR chaperone complexes in the cytoplasm are disrupted and the nuclear localization sequences are unmasked, resulting in an import of the ligand-bound GR into the nucleus (25). Localization of the GR appears to be dynamic, because cyclic administration, withdrawal of ligands in tissue culture as well as pulsed GC treatment of adrenalectomized rats lead to a cyclic localization between the nucleus and the cytoplasm. This GR shuttling is associated with cyclic GR target gene expression over time (26).

Within the nucleus, the ligand-activated GR binds to palindromic response elements of the DNA [GC response elements (GREs)] as homodimers in a head-to-tail fashion via hydrophobic motifs present in the LBD (27) and interactions by the DBD (28). The DBD consists of two zinc fingers. The amino terminal zinc finger of the GR molecule makes contact with the DNA. The D-loop adjacent to the second zinc finger motif interacts with the D-loop of the dimerization partner (28, 29). So far, crystal structures of the entire GR bound to DNA are lacking, leaving the possibility open that additional dimer interface exists. Recently, it was elegantly demonstrated by structural, biochemical and cell-based analyses that variations in the GREs result in differential GR conformational changes that affect recruited coactivators, leading to different transactivation functions on different GC target genes (30). In addition, experiments by the Hager group demonstrated that a preoccupied GR binding site by the GR helps to assist binding of additional GR molecules that exchange in a dynamic fashion (31). Transactivation of transcription is dependent on the AF domains of the GR. The AF-1 domain is responsible for transcriptional activation and association with basal transcription coactivators in a ligand-independent manner by interacting with ATPase BRG1 and recruiting histone acetylases like p/CAF and CBP/p300 (32). In contrast, the AF-2 domain with its helix 12 acts in a ligand-dependent manner by recruiting coactivators from the p160 family (e.g., SRC-1, TIF2/GRIP1) (Figure 1A). These coactivators possess histone acetylase activity and thereby help to open chromatin to allow transcriptional activation (33, 34).

Binding to DNA, however, can also lead to repression of the transcription of genes. Negative GREs (nGREs) were initially described as palindromic sequences separated by a three-base pair spacer and being present only in a few genes, such as POMC, prolactin, α-fetoprotein, CRH, osteocalcin, interleukin 1β, and proliferin (35). However, it was recently reported that additional nGREs exist as inverted repeats with none, one, or two spacing base pairs, leading to recruitment of corepressors, such as silencing mediator for retinoid and thyroid receptors and nuclear receptor corepressor (36).

Recent genome-wide binding studies of the GR in mammalian cells have revealed that binding motifs for the GR might be more diverse than previously anticipated, which explains in part the diverse response of genes dependent on the cellular context (37). In particular, cell-type-specific predetermined open chromatin sites in the absence of ligand revealed by genome-wide DNAse I hypersensitivity profiling seem to determine the majority of GR occupancy in the presence of hormone (38). To a minor degree, the occupancy of GR binding motifs is determined by binding sites for certain other transcription factors – again, dependent on the cell type as revealed by the comparison of a mammary cell line with a pituitary cell line (38).

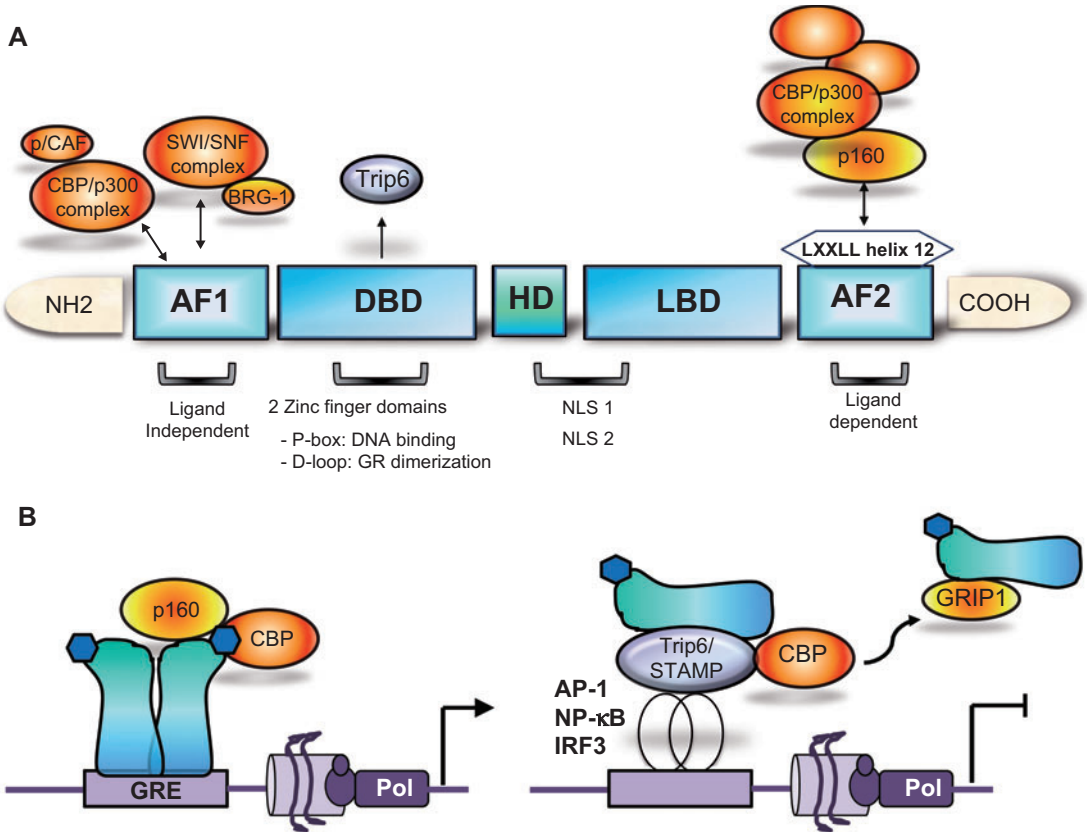

Figure 1 Primary structure of the glucocorticoid receptor (GR).
(A) The transactivation domain AF-1 is followed by the DNA-binding domain (DBD) with two zinc fingers involved in DNA binding (P-box) and dimerization of the receptor (D-loop), a hinge domain (HD) with two nuclear localization sequences (NLS), a ligand-binding domain (LBD), and a C-terminal transactivation domain AF-2. Coactivators with histone acetyltransferase activity such as CBP/p300, ATP-dependent chromatin remodeling complexes like switch/sucrose non-fermenting (SWI/SNF) proteins enhance the transcriptional activator function of the receptor in a ligand-independent manner. The AF-2 domain has a ligand-dependent coactivator function that recruits coactivators with the LXXLL motif in helix 12. Those coactivators belong to the p160 family, which further recruit multiple coactivators among the CBP/p300. Integrators such as TRIP6 interact with the DBD to mediate transrepression of AP-1. (B) The GR dimer binds to the palindromic glucocorticoid responsive elements (GRE) in regulatory sequences of GR target genes and facilitates transcription by recruitment of coactivators (p160, CBP). The GR as a monomer influences the activity of pro-inflammatory transcription factors like AP-1, NF-κB, and IRF3 by tethering mechanism with co-integrators (Trip6, STAMP). The liganded GR also could sequester GRIP1, usually, a coactivator for IRF3, from these transcription factors. By this mechanism, the pro-inflammatory gene activity is repressed or modulated.

Tethering of the monomer GR to other transcription factors

Another major mode of gene regulation is the association of GR as a monomer with pro-inflammatory-acting transcription factors [AP-1 (39–41), NF-κB (42, 43), IRF3 (44–46), STAT proteins (47), CREB, NFAT, T-bet, and GATA3 (48–50)] and thereby modulating their activity and target gene expression. Therefore, this *tethering* mechanism is also considered to be a *transrepression* mechanism.

The cointegrator proteins SRC1, TIF2-associated-binding protein (STAMP) (51), thyroid hormone receptor interactor 6 (TRIP6), GR-interacting protein 1 (GRIP-1), and positive transcriptional elongation factor b (P-TEFb) had been shown to be decisive to ensure transrepression (45, 46, 52, 53) (Figure 1B). Loss-of-function studies also point to a role of tumor suppressor protein p53 being involved in transrepression by an unknown mechanism (54).

Recent genome-wide studies by Rao et al. (55) measuring p65/NF-kB and GR occupancy on the DNA of TNF and GC triggered HeLa cells revealed a more detailed picture of the crosstalk of those two transcription signaling pathways. Both DNA-binding-dependent mechanisms utilizing partially pre-occupied DNA sites for GR and p65 as well as presumably DNA-binding-independent mechanisms of the GR in TNF and GC triggered cells seem to be involved. More genome-wide studies of inflammatory acting cells with GR mutations unable to dimerize (see below), for example, are required to resolve the mechanisms more comprehensively.

Contribution of the molecular functions of the GR therapeutic and side effects in steroid therapy

Because pro-inflammatory genes under the control of AP-1 and NF-κB can be repressed by the GR monomer, there is a long-standing hypothesis that selective-acting GR-modulating compounds that address the monomer function of the GR represent selective anti-inflammatory drugs with fewer side effects (56). Mouse models had been developed to prove the relevance of this concept.

Two different types of modification of the GR allele in the mouse were designed to achieve these goals. First, conditional GR knockout mice were generated, harboring a GR allele with loxP sites flanking exon 3 (8) or exon 2 (57), so-called GRflox mice. GRflox mice were crossed with transgenic mice expressing Cre recombinase in a cell-type-specific manner in adult mice to reveal the critical cell types involved in an organismic response to GCs (58). Second, GR^{dim} mice, a knock-in strain carrying a point mutation in the dimerization interface of the DBD encoded in exon 4 (59), were characterized to abrogate dimerization-dependent regulation of GRE-containing genes but allow DNA-binding-independent tethering mechanisms to AP-1 and NF-κB (29, 43, 59, 60). Interestingly, this germ line mutation has been shown to rescue a number of defects observed in complete knockout mice (14), such as lung maturation, development of the adrenal medulla, and thus perinatal lethality (59). Gene expression profiling revealed that the induction of metabolic genes is strongly reduced, but a subset of genes is regulated in a similar manner in the liver of wild-type and GR^{dim} mice following systemic prednisolone treatment (61). The differential regulation of genes by GCs in GR^{dim} mice makes these mice very suitable for studies determining the molecular mechanism in disease models where GCs are applied. This is of particular importance for functional target genes of the GR in therapeutic and side effects of GC application.

GC effects on skin inflammation

A classic animal model of acute irritant inflammation in the skin is phorbol ester-induced ear inflammation. It is characterized by edema formation, swelling and increased vascular permeability, influx of neutrophilic granulocytes, and mononuclear cells into the skin and can be determined by an increase of ear thickness. Topical treatment with GCs severely suppresses this inflammation in wild-type and GR^{dim} mice (62). Thus, the dimerization-deficient GR is sufficient to mediate an anti-inflammatory response.

In contrast, contact allergy, which can be modeled by contact hypersensitivity (CHS) in mice, requires GR dimerization for anti-inflammatory effects by GCs (63). GR^{dim} mice display a complete refractory response toward GCs, although some cytokines, such as TNF-α, are sufficiently suppressed. IL-1β, MCP1, and IP10 fail to be repressed in GR^{dim} mice. Using tissue-specific GR knockout mice in the CHS model, it has been revealed that the GR in keratinocytes and the GR in T cells are dispensable for GC treatment. Only mice lacking the GR in myeloid cells ($GR^{LysMCre}$) are resistant to GC therapy (62).

Thus, in different types of skin inflammation, different requirements of GR functions are involved in the anti-inflammatory activity of GCs.

Systemic inflammation: septic shock

Usually, inflammatory responses against infections are counterbalanced by a resolution of this inflammation, where endogenous GCs are considered to participate. Hence, patients with adrenal insufficiencies (64) have a decreased survival rate of septic shock as well as rodents subjected to adrenalectomy (12). Moreover, mice lacking the GR in macrophages are highly sensitive to septic shock induced by a bolus injection of lipopolysaccharides (LPS) (9). Their enhanced lethality is explained by an impaired expression of dual specific phosphatase 1 (DUSP1/MKP1) in macrophages by endogenous GCs, leading to a suppression of p38 activity, thereby blunting TNF-α expression. However, suppression of macrophage activity by GCs during this disease appears to occur on different levels. First, the degree of suppression of pro-inflammatory-acting MAP-kinases depends on the type of involved toll-like receptors with respective adaptors (44, 65). Second, GR dimerization-dependent functions in these cell types appear to be decisive, because macrophages from GR^{dim} mice have a less stringent efficiency to suppress cytokines (63). Furthermore, they are largely resistant to changes in cell shape, NO synthesis, and surface expression of classic activation markers (66) and fail to up-regulate GC and LPS synergistically regulated genes. Moreover, GR^{dim} mice display an enhanced lethality in various septic shock models (66). Interestingly, in these mice, TNF-α is not misregulated in septic shock, but IL-1β and IL-6 levels are increased. Indeed, inhibition of IL-1 activity by recombinant IL-1 receptor antagonist administration rescues $GR^{LysMCre}$ mice completely and GR^{dim} mice partially from death after LPS administration (66). This demonstrates that IL-1β is an important target of endogenous GCs protecting against septic shock.

GCs have been reported not only to diminish pro-inflammatory features of macrophages but also to induce 'alternatively' activated macrophages (67). These have been implicated in the resolution of inflammation by expressing IL-10 with anti-inflammatory activity and enhancing the expression of scavenger receptors, such as CD163, FcR (CD16 and mannose receptor) (68). Therefore, the clearance of apoptotic cells (69), a crucial process for the resolution of inflammation, is enhanced. Studies of genome-wide gene expression and phagocytosis in GC-exposed human monocytes – the precursors of macrophages – have corroborated the GC-induced 'anti-inflammatory', presumably alternative, phenotype (70). Furthermore, GCs enhance the survival of anti-inflammatory-acting monocytes by up-regulation of the A3 adenosine receptor to prevent apoptosis (71).

Thus, myeloid cells, in particular, monocytes/macrophages, play a key role in systemic inflammation, which needs to be addressed by GCs in order to prevent it.

GC action in suppression of inflammation in models of autoimmunity

Multiple sclerosis is the most prevalent autoimmune disease of the central nervous system. High-dose GC therapy is applied during acute phases of the disease; however, accompanied complications and sometimes incomplete recovery can occur (72). Using an animal model, experimental autoimmune encephalomyelitis (EAE), Wust et al. (73) and Schweingruber et al. (74) could demonstrate that GC action in T cells and myeloid cells is decisive in a therapeutic setting. $GR^{LysMCre}$ mice were found to be curable by free accessible GCs, whereas GR^{LckCre} mice, lacking the GR in T cells, displayed an earlier onset of the disease and were completely resistant to GC application, remaining heavily inflamed (73). In contrast, when GCs encapsulated in liposomes that are taken up by phagocytotic cells, macrophages were identified to be the main targets (74). This impressively demonstrates that dependent on the formulation of corticosteroids, different cell types could become critical for anti-inflammatory action. It remains a challenge to underpin the exact mechanisms behind these differences.

Concerning the involvement of GR dimerization, no studies have been performed yet, but it is noteworthy that 'compound A' (CpdA), a GR-selective ligand favoring monomer GR function, has been reported to be successful in suppressing EAE (75, 76).

GC effects on T cells and their precursors are long established. Double positive ($CD4^+$, $CD8^+$) T cells are very vulnerable to GC-induced apoptosis. GR^{dim} mice fail to show apoptosis of double positive cells (59). Potential target genes of the GR are the proapoptotic proteins Puma and Bim (77, 78). Mice lacking the GR in T cells do not display altered thymocyte subsets or TCRβ expression (57, 79), excluding a role of the GR during thymocyte selection. Interestingly, GCs produced by the thymus stroma and/or epithelial cells appear to mediate post-pubertal thymus involution in male mice dependent on androgen action (80, 81).

GCs can suppress activation-induced cell death of peripheral T cells (82, 83) and inhibit T cell activation, suppress a plethora of cytokines, induce apoptosis, and shift from Th1 to Th2 cells, reviewed elsewhere (84). The actions of GCs on Th17 cells and regulatory T cells (T_{regs}), both important modifiers of autoimmune diseases, have been less studied to date. T_{regs} are potent suppressors of autoinflammatory T cell responses and have been reported to be elevated upon GC treatment (85, 86). Despite the observation that T_{regs} primed *in vitro* by GCs impede EAE in mice after transfer, endogenous T_{reg} numbers are reduced during GC therapy of EAE (73). Nonetheless, a reduction of IL-17 cells accompanies GC treatment in EAE, indicating a potential mechanism for GR action to suppress inflammation in this model.

Inhibition of IL-17-producing cells by the GR dimer appears to be in part also important for the treatment of another autoimmune disease, rheumatoid arthritis, as revealed by the resistance of GR^{dim}, GR^{LckCre}, and IL-17 knockout mice to GC treatment in antigen-induced arthritis (87).

Taken together, the GR in T cells, most likely in the IL-17-producing subset, is critical for the suppression of inflammation in the hitherto analyzed models of autoimmune diseases. However, myeloid cells may also play a role depending on the availability of GCs.

Side effects

The most prominent side effects of GC excess, either applied pharmacologically or being present in disease conditions, are muscle weakness, hyperglycemia, hepatic steatosis, insulin resistance, and bone loss (10).

Muscle weakness GC-induced muscle loss occurs via the GR in muscle (88), in part by up-regulation of the muscle degradation initiating E3-ubiquitin ligase Murf1/Atrogenin (89). Despite the requirement of GR dimerization to up-regulate Murf1/Atrogenin, muscle loss is not affected in GR^{dim} mice. This could be due to non-genomic effects, because overexpression of a nuclear localization defective GR in muscle fibers of muscle specific knockout mice could confer decrease of muscle fiber size (88). Paradoxically, also, Murf1/Atrogenin was in these mice still induced, in contrast to GR^{dim} mice (89). Thus, more unrecognized GC-regulated genes contribute to muscle weakness. Furthermore, the GR in muscle contributes to diabetes-induced muscle wasting (88).

Insulin resistance Insulin resistance as one of the major complications is rapidly evoked by GC administration, which is in part due to the suppression of insulin secretion from β cells by GCs (90, 91). Moreover, mice overexpressing the GR in β-islets display an increased insulin resistance (90). Furthermore, GCs have direct effects on insulin receptor signaling by affecting the expression of insulin receptor substrate 1 and 2, reduction of the activity of PI3K, and thus Akt phosphorylation, which may be mediated by ceramides. Rodents with impaired ceramide synthesis are rendered resistant to GC-induced insulin resistance and do not show reduced Akt phosphorylation (92).

Nonetheless, loss of GR function in the liver of mice illustrates features of the role of endogenous GCs in metabolism. In the liver, GCs, together with glucagon, regulate glucose output, which is antagonized by the actions of insulin (93). Mice lacking the GR in the liver show a strong hypoglycemia after fasting (94), which is accompanied by a failure of the up-regulation of mRNA encoding key gluconeogenic enzymes tyrosine aminotransferase and phosphoenol pyruvate carboxykinase. Both are characterized by GREs in the promoters and require dimerization-competent receptors for up-regulation of mRNA by GCs (59). Indeed, unpublished work in our laboratory favors GR dimerization-dependent processes in GC-induced glucose intolerance.

Hyperglycemia is further augmented by the breakdown of protein and fat stores to enhance the substrates for gluconeogenesis (93). Long-term GC treatment up-regulates PPARα (95), which is involved in insulin resistance. PPARα-deficient mice display a reduced insulin resistance after 5 months of dexamethasone treatment. Interestingly, interruption of vagal

nerve innervation in the liver reduces insulin resistance of GC-treated wild-type mice, but not of PPARα-deficient mice (96). How PPARα activity links vagal nerve innervation to GC-induced insulin resistance and whether this mechanism applies to short-term GC exposures require investigation. Very recent liver X-receptor β was recognized as a mediator for GC-induced insulin resistance by participating in gluconeogenetic gene regulation (97). Thus, mechanisms involved in GC-induced insulin resistance are complex and require further comprehensive investigations.

Lipid metabolism Within the liver, GCs exert profound effects on lipid metabolism and contribute to liver steatosis via the GR at least in obese mice (96). In particular, they are involved in the elevation of plasma non-esterified fatty acids, induction of lipolysis in fat tissue, and therefore enhanced triglyceride synthesis, which is in combination with a decrease of fatty acid oxidation; lipid accumulation in the liver triggers for hepatic insulin resistance (93). GCs exert profound effects on fat tissue. Redistribution of peripheral fat, which is poorly understood, and gain of visceral fat are augmented by GCs. Lipoprotein lipase is up-regulated upon GC exposure; the uptake of non-esterified fatty acids and triglycerides into adipocytes is augmented (93).

In addition, the generation of adipocytes from mesenchymal stem cells is facilitated by GR activity. Further adipogenic differentiation involves the actions of several transcription factors, most importantly, those of the CCAAT/enhancer-binding family of proteins (C/EBPs) and PPARγ. C/EBPβ and C/EBPδ are early induced genes that precede the expression of the key adipogenic transcription factors C/EBPα and PPARγ for the terminal differentiation of adipocytes (98).

Derepression of C/EBPβ by the GR ligand domain at the promoter of C/EBPα in the preadipocytic model cell line 3T3-L1 (99, 100) as a non-genomic mechanism had been proposed. However, genomic effects of the GR appear to play a major role. Very recently, Siersbæk et al. (101) studied the transcription factor recruitment to open chromatin sites during adipocyte differentiation in 3T3-L1 cells. Here, they identified that the GR in concert with retinoid X receptor, STAT5α, C/EBPβ, and δ works as pioneering factors to prepare transcription factor hotspots a few hours after differentiation has been initiated. These factors in later differentiation events facilitate in part PPARγ binding (101). Additionally, dexamethasone stimulates C/EBPδ expression and subsequent PPARγ and C/EBPα expression in 3T3-L1 cells (102). Recently, we found that GR dimerization is required for this process (103). Mouse embryonic fibroblasts of GR^{dim} and GR knockout mice, cultured under adipogenic conditions, display a strong reduction of adipogenic differentiation capacity. GR^{dim} and GR knockout cells fail to up regulate KLF15, which is required for adipogenesis *in vitro* (103). However, whether the generation of de novo fat involves the GR *in vivo* requires further investigation.

Osteoporosis GC-induced osteoporosis (GIO) is a major side effect of steroid treatment: 25% of all clinical osteoporotic incidences in clinics are associated with high GC intake or GC excess (104, 105). The bone loss observed in GIO is due to an adverse imbalance of bone formation by osteoblasts and bone resorption by osteoclasts. Systemic effects of GCs such as lowering of calcium levels and those of anabolic-acting hormones, for example, sex steroids and growth hormone, can contribute to bone loss to a certain extent (106). However, direct effects on bone cells have been shown to be crucial in causing bone loss.

GCs are potent inducers of osteoclastogenesis-promoting receptor activator of NF-κB ligand (RANKL) and suppress the osteoclast inhibitor osteoprotegerin (OPG) (107). Conflicting results exist over GC effects on osteoclast activity in mouse models. Whereas increased resorption has been reported in GC-treated Balb/c mice (108), other studies have reported decreased resorption (approx. 20%–30%) in mice of different backgrounds (17, 109). Jia et al. (109) suggest that GCs enhance the lifespan of mature osteoclasts, whereas osteoclastogenesis from osteoclast progenitors is inhibited. The inhibition of osteoclastogenesis by pharmacological GC concentrations observed in co-cultures of osteoblast and osteoclast precursors is dependent on GR expression in both cell types (17). This observation is in line with the observed decrease of resorption *in vivo* reported by Rauch et al. (17) and Kim et al. (110). The cell-autonomous inhibition of osteoclastogenesis by GCs is explained by impairment of the reorganization of the cytoskeleton (actin rings for resorptive osteoclast activities) (110). Importantly, total inhibition of resorption by bisphosphonates or by RANKL inhibitory antibodies in humanized RANKL knock-in mice ameliorates GC-mediated bone loss (108).

Recently, it was shown that the GR in osteoblasts is crucial for GC-induced bone loss. Mice lacking the GR in osteoblasts ($GR^{Runx2Cre}$ mice) are resistant against GC-mediated inhibition of bone formation and loss of bone mass (17). Inhibition of bone formation is accompanied with reduced osteoblast and osteocyte numbers (111). The reduction of osteoblast numbers could be due to inhibition of proliferation, induction of apoptosis or inhibition of differentiation.

The proliferation of osteoblasts can be inhibited by GCs by antagonizing the Wnt pathway (112), GC-induced MKP1/DUSP1, leading to a reduction of mitogenic signaling (113) that requires GR dimerization (17). Nevertheless, GR^{dim} mice have impaired bone formation upon GC exposure, indicating that effects of GCs on proliferation are involved in GC-induced bone loss to only a minor extent (17). Apoptosis of osteoblasts and osteocytes is observed after prednisolone treatment (114), is independent of GR dimerization (17), is accompanied with caspase 3 activation (115), has induction of the pro-apoptotic protein BAX (116) and might depend, in addition, on the activation of the FAK-related kinase Pyk2 (117). Furthermore, an overall bone loss is still displayed in mice with impaired GC activity and consequently reduced apoptotic rates in late differentiated osteoblasts and osteocytes *in vivo* (118), indicating that induced apoptosis is not the only mechanism of GC-induced bone loss.

High dosage of GCs impairs the differentiation capacity of primary osteoblasts by about 70% to 90% (17) and is

accompanied by reduced differentiation marker gene expression (119). Following reduced differentiation, osteoblast function (collagen production) declines (120) *in vitro* and *in vivo* and, consequently, bone formation is reduced. This GC effect is absent in mice specifically lacking the GR in osteoblasts ($GR^{Runx2Cre}$), but not GR^{dim} mice (17) and prevents bone loss only in $GR^{Runx2Cre}$ mice. Taking these findings together, it can be concluded that the inhibition of differentiation is a major mechanism of GIO (17).

GCs can affect differentiation by engagement of multiple mechanisms and factors, including suppression of BMP/TGFβ signaling (121, 122), growth hormone/IGF-1 activity and affecting AP-1-dependent LIF and particular IL-11 expression by the GR monomer in osteoblasts. IL-11 is a potent inducer of osteoblastogenesis *in vitro* and *in vivo* via the Jak2/STAT3 pathway, and when applied in excess, it can counteract the GC-induced suppression of osteoblast differentiation (123–125).

Novel criteria for selective GR modulators for therapeutic efficacy and prevention of osteoporosis

Based upon the observation that the monomer GR can repress the activity of pro-inflammatory transcription factors, in particular, AP-1 and NF-kB, selective ligands for the GR have been developed on the principles that they should retain monomeric function of the GR but disrupt dimerization functions. The so-called selective GR modulators or activators (SEGRM, SEGRA) (2, 126) should maintain immune suppression but avoid side effects.

In the early years, potential compounds were screened for their ability to suppress a cytokine promoter and for their inability to transactivate GRE-containing promoters. Selected compounds could dissociate between transactivation and transrepression of the GR *in vitro*. However, some of them exerted side effects (127, 128), such as weight loss, shrinkage of the thymus, and bone turnover (128) or the induction of gluconeogenetic enzyme genes in liver cells (129). Nonetheless, for local topical application to treat inflammatory skin diseases, the non-steroidal compound ZK245186 looks to be a promising candidate to maintain anti-inflammatory efficacy and to avoid brittleness of the skin (130) and is currently in clinical trials. Studies using cell-type-specific and function-selective knockout mice have revealed different GR mechanism requirements for the therapeutic and side effects of classic GR ligands (Figure 2). For some anti-inflammatory activities, such as irritant skin inflammation, a GR defective in dimerization is able to perform full anti-inflammatory activity. In other inflammatory types, contact allergy, septic shock, and arthritis, dimer-dependent mechanisms are required. Furthermore, the activity of the GR in distinct cell types appears to be critical. Side effects also utilize GR dimer-dependent and GR dimer-independent mechanisms. The induction of hyperglycemia appears to be GR dimer dependent (Rauch and Tuckermann, unpublished data) as expected, whereas GIO occurs in the absence of GR dimerization and a discrimination between interference of NF-kB vs. AP-1 might be important to protect osteoblast differentiation while still promoting some anti-inflammatory activity.

Our analysis of the activity of the GR ligand CpdA on bone cells demonstrates that these criteria can be met in osteoblasts (123). CpdA displays potent anti-inflammatory actions in collagen-induced arthritis (131). In addition, CpdA is capable of suppressing pro-inflammatory cytokines, such as CXCL10 and IL-6, and does not influence the RANKL/OPG ratio in osteoblastic cell lines and primary cells (123, 132), whereas expression of *Il11* and osteoblast differentiation are unaffected by CpdA. Finally, mice with collagen-induced arthritis receiving an immunosuppressive dose of CpdA have strikingly higher serum osteocalcin levels compared with dexamethasone-treated animals (123). Therefore, although CpdA has a narrow therapeutic window, such compounds with optimized pharmacology could be of major clinical use to suppress inflammatory bone diseases and maintain bone integrity.

This example demonstrates that for the future screening of optimized GR ligands, a more specific screening approach should be undertaken. In particular, cellular readouts need to be developed, which resemble therapeutic and side effects *in vivo*. The most promising candidates will be required to avoid particular side effects but maintain anti-inflammatory efficacy. Moreover, selective GR ligands may only be successful in certain types of inflammation, as revealed from studies with conditional mutant GR mice.

Expert opinion and outlook

Advanced understanding about the molecular basis of the physiological effects of GCs was obtained by the analysis of conditional knockout and knock-in mice for the GR. The requirement of the GR in T cells for immune suppression in a model of multiple sclerosis, but in myeloid cells for models of contact allergy and septic shock (66), could be unequivocally demonstrated. In part, the immune suppressive effects are dependent on GR dimerization. Also, side effects of GC medication seem to employ different modes of GR activity. For GIO, interaction of the GR monomer with AP-1 is required, whereas for induction of adipogenesis, a dimerization competent GR is necessary.

Albeit these analyses are far from being complete, it becomes evident that for different inflammatory disease models and for different side effects, specific modes of GR action in particular cell types are involved. Further insights of the tissue-specific molecular mechanisms of the GR will be obtained in the near future by combining the physiological analysis of the transgenic mouse models with the genome-wide determination of gene regulatory activity and functional interference of GR target genes in a tissue-specific manner. The comprehensive understanding of GR biology will give novel rationales for the development of specific GR modulators that may reduce side effects and keep anti-inflammatory efficacy in particular disease conditions.

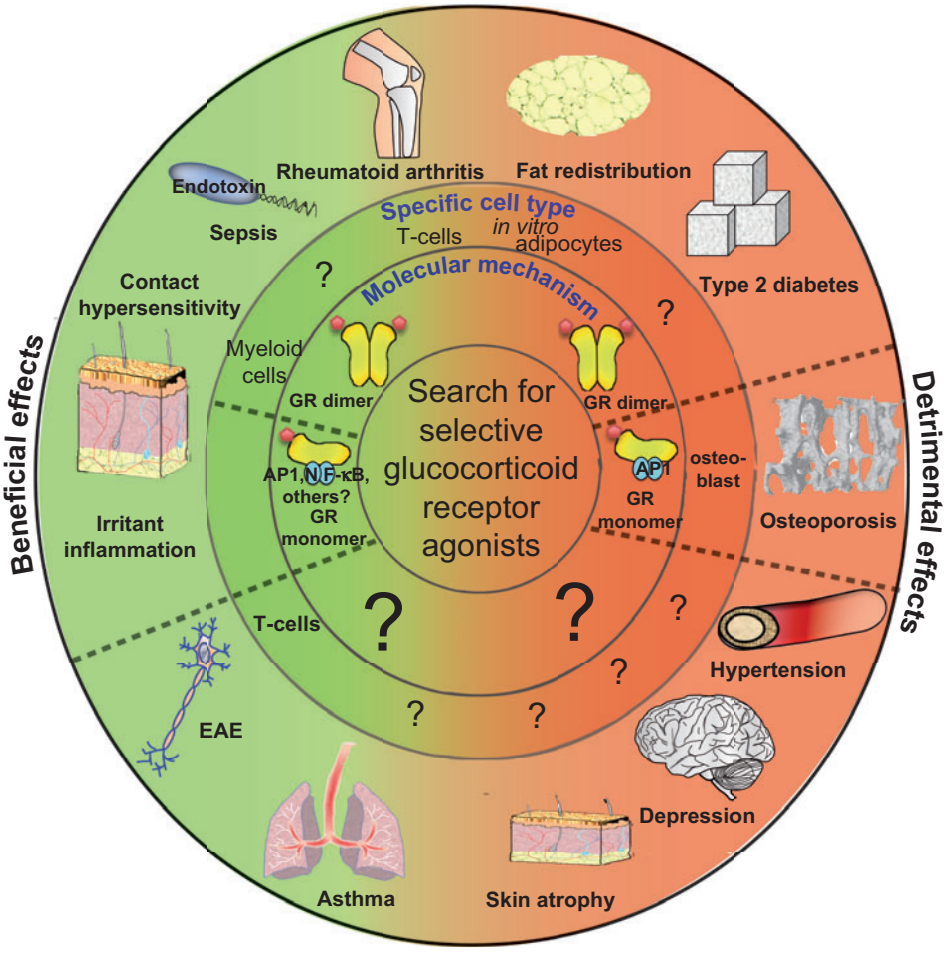

Figure 2 Different physiological processes in steroid therapy require cell-type-specific modes of GR action.
Shown is a graphical summary of the results from analyses of GR dimer-deficient and cell-type-specific GR knockout mice in beneficial (left) and detrimental effects (right) of GC therapy. For beneficial anti-inflammatory effects of GCs (left), GR dimerization is required in animal models for arthritis, sepsis, and contact allergy. For irritant inflammation, the monomer GR activity is sufficient; however, the involved interacting partners remain to be identified. The GR in T cells is required for the immunosuppressive effects in an animal for model experimental autoimmune encephalomyelitis (EAE) and arthritis, whereas cell-type requirements for asthma have not been demonstrated. Detrimental side effects (right) of the GR dimer-dependent processes in adipocytes are important for differentiation and possibly for fat redistribution. Type 2 diabetes seems to be GR dimer dependent, which has to be further investigated. Monomer GR-AP-1 interaction is critical for GC-induced osteoporosis. Models for hypertension, brittleness of the skin and depression remain to be analyzed with conditional GR knockout mice. Selective GR modulators should fulfill the molecular requirements, specifically for individual inflammatory diseases, and then should exert distinct capacities to reduce side effects.

Highlights

- GR acts by different mechanisms to suppress inflammation dependent on the disease type.
- Side effects may depend on GR dimerization but also employ GR monomer dependent activities as in the case of GIO.
- Molecular mechanisms of GR activity in a genome-wide manner are just becoming uncovered and reveal novel DNA recognition sequences for direct or indirect binding of the GR. How cell-type specificity governs gene regulation by the GR remains a challenge in the field and needs to be addressed.
- Selective GR modulators addressing cell-type-specific mechanisms of the GR are required for reduced side effects in the therapy of particular inflammatory diseases.

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