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Review

A novel aspect of the anti-inflammatory actions of glucocorticoids: inhibition of proximal steps of signaling cascades in lymphocytes

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Abstract. Glucocorticoid hormones are effective in inhibiting inflammatory responses, but the mechanisms that confer this action have not been completely elucidated. The prevailing view is that these compounds inhibit novel gene transcription regulated by the nuclear factor kappa B and/or activator protein-1 transcription factors. In the last few years, several reports have shown that glucocorticoids can also block signal transduction in lymphocytes at an early, postreceptor step, suggesting novel molecular targets for these hormones. These data will be briefly reviewed and the possible in vivo relevance of these findings discussed, with particular emphasis on T cell development.

Key words. Glucocorticoids; lymphocytes; signal transduction; membrane rafts.

Introduction

Glucocorticoids (GCs), the final products of activation of the hypothalamic-pituitary adrenal axis, regulate numerous activities in vivo, including metabolism, growth, neuronal function and virtually every aspect of the immune response [1]. The profound effects of these compounds on the immune response have led to their widespread use as antiinflammatory and immunosuppressive agents to prevent graft rejection, and to treat autoimmune, allergic and inflammatory diseases [2, 3]. However, despite their broad therapeutic use, the precise mechanism by which GCs exert their immunomodulatory actions in vivo remains difficult to identify, due in part to the multiplicity of the biological effects they mediate on a large set of lymphoid cells.

Due to their lipophilic nature, glucocorticoids diffuse freely through the cell membrane and bind to specific cytosolic receptors (GRs) expressed by virtually every lymphoid cell, albeit with some heterogeneity [4]. Hormone-receptor complexes translocate to the nucleus and regulate, either positively or negatively, the expression of numerous genes [5]. The most widely accepted view is that GCs mediate their potent antiinflammatory and immunomodulatory actions through the inhibition of the activity of several transcription factors involved in the regulation of cytokine gene expression such as nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) [6, 7]. GCs are known to inhibit the production of a large number of cytokines including interleukin (IL)-1, IL-2, tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ), produced by both lymphocytes and mononuclear cells [8–11]. The precise molecular mechanisms by which GCs affect the activity of transcription factors are a still controversial [6, 12–14]. The consensus, however, from

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these studies is that GCs do not affect generation of transcription factors but rather inhibit their function through three possible mechanisms, including (i) direct inhibition of gene transcription secondary to the binding of ligand-GR complexes to a negative regulatory element in the promotor region [5, 15]; (ii) protein-protein interactions, a mechanism whereby ligated GRs bind to transcription factors and impede their normal transactivating function. This action of GCs is independent of their ability to activate or repress transcription, and genes lacking a GR response element may be sensitive to this negative influence of GC [16–20]; (iii) increased expression of IκB, a protein able to bind and sequester NF-κB in the cytoplasm [21, 22].

In all instances, these studies led to the prevailing concept that GCs affect lymphocyte activation by interfering with a distal step of the signaling cascade initiated upon Ag stimulation. Moreover, these data suggest that the antiinflammatory effects of GCs are mostly related to their negative regulation of cytokine-gene transcription, whereas the side effects result from their transcription-promoting properties. These topics have been recently discussed in a series of recent reviews [14, 23] and will not be treated here. A growing body of data that will be briefly summarized below suggest, however, that GCs may inhibit immune responses independently of their known effect on cytokine gene transcription. These data are mostly related to T cell differentiation events in the thymus and in the periphery, and are best explained by assuming that in addition to affecting gene transcription, GCs may affect a proximal step of the signaling cascade initiated in lymphoid cells by antigen or cytokines.

Glucocorticoids and cell fate decision in the thymus and in the periphery

Although the adrenals represent the major source of GCs (cortisol in humans, corticosterone in rodents), it has recently been shown that that the thymic epithelium and possibly thymocytes themselves produce steroids, suggesting a potential role of GCs in the control of T cell development [24–27]. In agreement with this hypothesis, downregulation of GC receptors on thymocytes and/or pharmacological inhibition of glucocorticoid synthesis have been shown to affect thymic differentiation in vivo [28–31]. Earlier observations performed on thymocytes and T cell hybridomas may provide a clue on the mechanism by which GCs may regulate T cell differentiation in the thymus. Indeed, whereas GCs or signals delivered through the T cell receptor (TCR) alone result in apoptosis, simultaneous exposure of thymocytes to both stimuli results in cell survival [32, 33]. This phenomenon referred to as ‘mutual antagonism’, suggest that GCs may inhibit negative signaling through the TCR and allow the survival (positive selection) of cells displaying a moderate self-reactivity [23]. The model posits that GCs cannot overcome negative signaling issued by TCR binding with high affinity to self-antigens, thus ensuring negative selection of potentially harmful autoreactive T cells. The mechanism by which GCs antagonize TCR signals during T cell development is presently unknown but seems to be unrelated to the ability of GCs to induce apoptosis or to modulate cell death through Fas/Fas ligand signaling [34].

The identification of functionally distinct CD4 helper subsets producing distinct patterns of cytokines has provided an important insight into immune regulation. Indeed, Th1 cells secreting INF-γ promote inflammatory and cytotoxic responses, whereas Th2-derived cytokines (IL-4, IL-5 and IL-10) favor the humoral response and exert antiinflammatory properties by inhibiting Th1 responses [35, 36]. Numerous studies have suggested that Th1- and Th2-like cells originate from a common ‘Th0’ precursor and that this developmental choice is under the influence of the environment (including antigen dose, cytokine milieu and nature of the antigen presenting cell) [37–40]. The analysis of the effect of GCs on T helper subset development has led to some intriguing results. Indeed, studies mostly performed in vitro have led to the conclusion that GCs inhibit the secretion of both Th1 (IL-2, IFN-γ) and Th2 (such as IL-4 and IL-5)-derived cytokines [8, 9, 41–43]. Expression of these cytokine genes is mediated by shared transcription factors such as AP-1, NF-κB and nuclear factor of activated T cells (NF-AT), which are all sensitive to the negative influence of GCs. In particular, it has recently been shown that GCs can inhibit the secretion of the Th2-derived cytokine IL-4 by interfering with NF-AT-dependent transactivation of the proximal IL-4 gene promotor [43]. These findings are, however, difficult to rationalize with numerous observations performed on rodent and human models indicating that GCs minimally affect Th2 responses both in vitro and in vivo [44–46]. In a seminal study, R. A. Daynes and B. Araneo demonstrated that GCs differentially affect Th1 and Th2 responses. Using T cell lines able to secrete both Th1- and Th2-like cytokines, these authors demonstrated that under conditions where IL-2 production was reduced by GCs, IL-4 secretion was increased [47]. Numerous studies confirmed these early observations and led to the conclusion that whereas GCs display potent antiinflammatory activities by suppressing Th1-type cytokines (such as IL-2, IFN-γ and TNF-α), they often increase the production of Th2-derived cytokines such as IL-4, IL-10 and IL-13 [44–46]. The mechanism by which GCs may differentially affect Th1 versus Th2 responses is presently unknown but is difficult to relate to the inhibition of cytokine gene transcription, as GCs inhibit the transcription of many Th2 cytokines genes.

Developmental decisions during T cell development and following antigen encounter in the periphery appear to be regulated by the quantity and the quality of intracellular signals issued by antigen/major histocompatibility complex (Ag/MHC) engaged TCR complexes [48]. It is therefore tempting to speculate that GCs may affect T cell differentiation by inhibiting an early step in the TCR signaling cascade.

Receptor signaling and glucocorticoids

Antigen-specific receptors on T (TCR) and B (BCR) lymphocytes deliver intracellular activation signals upon recognition of the appropriate ligand [49–53]. These activated receptors initiate a cascade of phosphorylative events leading to the activation of downstream effector enzymes such as phosphoinositide-specific phosphodiesterases of the phospholipase C-γ (PLC-γ) subtype. Upon tyrosine phosphorylation, PLC-γ is recruited to the membrane, where it hydrolyses phosphatidylinositol (4,5)-biphosphate (PIP2) into the intracellular second messengers inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG), leading to an increase in intracellular calcium levels. Similarly, antigen-receptor-induced protein tyrosine kinase (PTK) activity is responsible for activation of the Ras/mitogen-activated protein kinase cascade. These signaling pathways finally activate a series of transcription factors (including AP-1, NF-AT and NF-κB) which ultimately lead to cellular responses such as proliferation and cytokine secretion [53].

Numerous independent observations have indicated that GCs can affect the early steps of signal transduction initiated upon antigen in both B and T lymphocytes. G. Dennis and colleagues demonstrated that dexamethasone (Dex) inhibited anti-Ig-induced B cell proliferation by affecting an early step of the signaling cascade [54]. In particular, both the generation of water-soluble inositol phosphates and elevation of intracellular calcium were inhibited by Dex. A key observation from this study was that cell proliferation induced by pharmacological agents bypassing the early step of receptor signaling [a combination of phorbol myristate acetate (PMA) and a calcium ionophore] was insensitive to GCs, suggesting that inhibition of receptor signaling rather than activation of transcription factors secondary to intracellular calcium elevation was the major target of GCs in B lymphocytes. These observations were extended to T cells in our laboratory. Incubation of murine T cell lines with Dex prevented the intracellular calcium increase induced by TCR aggregation [55]. GCs had no effect on calcium responses initiated by stimulation of a heterologous G-protein-coupled muscarinic receptor, suggesting that GCs did not affect calcium metabolism in these cells, but rather inhibited a membrane proximal step of the TCR-signaling cascade. Note that GCs inhibited IL-2 secretion by T cells stimulated by both a TCR ligand and by a combination of PMA and calcium ionophore, suggesting that GCs affect both a membrane proximal (preceding calcium influx) and a distal (secondary to calcium elevation) signaling step [our own unpublished observations].

Noteworthy, inhibition of TCR receptor signaling by dexamethasone required binding to the intracellular receptor, long term (>6 h) incubation and de novo protein synthesis, suggesting that these immunomodulatory effects of GCs required novel gene transcription.

Observations performed on the prototypic mast cells line RBL-2H3 are in agreement with the hypothesis that in addition to their well-characterized effect on gene transcription, GCs can inhibit an early post-receptor event. Dex was shown to inhibit receptor-induced inositol-phosphate formation and the early antigen-induced phosphorylation of several substrates in a GC-receptor-dependent manner [56]. In the same model system, Dex has been recently found to inhibit the antigen-induced increase in the kinase activity of a member of the mitogen-activated protein (MAP)-kinase family, the c-Jun N-terminal kinase (JNK), resulting in hypophosphorylation of c-Jun, a component of the transcription factor AP-1 [57, 58]. Although the JNK protein content in the cells was decreased by Dex, the rate of decrease in enzyme activity was much more prominent than reduction in protein content [58].

Notably, GCs have been found to inhibit the response of lymphoid cells to cytokines. Cytokines exert their effects by activating receptor-associated kinases of the Janus kinases family [59, 60]. These kinases phosphorylate specific tyrosine residues in the cytoplasmic domain of cytokine receptors, providing docking sites that are recognized by transcription factors of the signal transducer and activator of transcription (STAT) family. The prevailing view is that GCs inhibit cytokine-induced gene transcription following a physical interaction between STAT molecules and GC receptors [61]. A number of reports suggest, however, that GCs may inhibit cytokine receptor signaling at a membrane proximal step. GCs were found to inhibit signal transduction through the IL-2 receptor without affecting binding of IL-2 to membrane receptors [62], suggesting that inhibition occurred at a postreceptor level. In this study, tyrosine phosphorylation of several substrates was found to be inhibited following long-term incubation of activated human PBLs in Dex-supplemented media. Similarly, GCs have recently been found to inhibit IL-2-induced IL-5 secretion in allergen-specific human clones [42]. Notably, neither NF-AT nor NF-κB were significantly induced in the nuclear extracts of IL-2-stimulated T cells. Although the authors concluded that GCs inhibited IL-2-induced IL-5 secretion by affecting a still unknown transcription factor, these data are compatible with the notion that GCs downregulate IL-2R transmembrane signaling. Similar observations were per-

formed in T and NK cells in response to IL-12. Dex inhibited IL-12-induced IFN-γ secretion even though IL-12R expression and IL-12-induced Janus kinase (Jak) kinase phosphorylation remained unaltered [63]. In keeping with previous models, Dex inhibited IL-12-induced Stat4 tyrosine phosphorylation without affecting Stat4 protein expression.

Collectively, these data suggest that in addition to their well-described ability to modulate the activity of transcription factors, GCs may inhibit an early phosphorylative event of the signaling cascade initiated in lymphocytes by antigen or cytokines.

Receptor signaling, membrane microdomains and glucocorticoids

To gain insight into the mechanism by which GCs antagonize signals delivered by the TCR, our laboratory recently undertook a detailed analysis of the early phosphorylative events initiated upon TCR triggering [64]. This analysis revealed that incubation of murine T cell lines and developing thymocytes in Dex results in the hypophosphorylation of several intracellular substrates, including components of the TCR complex (the TCR ζ chain), LAT and the tyrosine kinase ZAP70 upon TCR stimulation. Surprisingly, however, neither the protein levels nor the in vitro enzymatic activity of the Src-family kinases involved in the early phases of TCR signaling were affected by GCs. In keeping with most studies described in the previous paragraph, GCs appeared to inhibit TCR signal transduction without affecting the intracellular protein levels of the TCR-associated kinases and their relative substrates.

Recently, the important role of glycolipid-enriched membrane microdomains (referred to as GEMs or detergent-insoluble lipid rafts) in cell signaling has been recognized (for review, see [65–69]). These GEMs are enriched in lipid-modified proteins such as tyrosine kinases of the *src* family, and adaptor protein such as linker for activation of T cells (LAT) [65]. Other molecules, including the TCR complex itself and the transmembrane protein phosphatase CD45, are excluded from these membrane subdomains [70]. Upon stimulation, however, components of the TCR/CD3 complex are recruited into the GEMs and phosphorylated on tyrosine residues by resident PTKs [71–73]. Although the physiological significance of this lateral microdomain organization of biological membranes is not clear, the confinement of signaling molecules to membrane subdomains suggests that these compartments function as platforms for the formation of multicomponent transduction complexes [74]. Accordingly, numerous studies performed on T lymphocytes have shown that raft integrity is required for effective TCR signal transduction [71–73, 75].

Based on the notion that steroids affect lipid metabolism [76], we hypothesized that Dex could affect the TCR signal transduction machinery by affecting the molecular composition of membrane rafts. Detergent-insoluble lipid rafts were isolated from both control and Dex-treated cells according to standard procedures. Steroids did not overtly affect plasma membrane microdomains, since GEMs enriched for both the GPI-linked CD90/Thy-1 protein and the ganglioside GM1 could be isolated from Dex-treated cells. GEMs from treated cells, however, displayed a marked decrease in the amount of p56lck, p59fyn and LAT normally associated with these submembrane structures [64]. These observations indicate that GCs affected the submembrane localization of transmembrane proteins (such as LAT) or tyrosine kinases normally found within lipid rafts. Dispersion of these signaling molecules in the soluble membrane fraction may impede an adequate juxtaposition of PTKs with their substrate, causing defective transduction of activation signals. We therefore propose that GCs can inhibit early phosphorylative events induced by antigen stimulation without affecting the protein expression profile of signaling molecules by modulating the physical relationship between important kinases and their substrates.

Adequate submembrane compartmentalization is required for optimal signaling by TCRs, BCRs, Fcε receptors and cytokine receptors [71, 72, 77–79], all of which have been shown to be sensitive to the membrane-proximal effects of GCs. Multiple tyrosine-phosphorylated proteins accumulate transiently upon BCR activation in detergent-insoluble membrane microdomains, and an intact raft structure was required for BCR-induced tyrosine phosphorylation of PLCγ2 and the induction of Ca(2+) flux [80]. Similarly, crosslinking of immunoglobulin (Ig)E-FcεRI complexes on RBL-2H3 mast cells causes their association with detergent-resistant membranes, and induces the spatially restricted activation of Syk and PLCγ1 [77]. Recently, a role of membrane lipid rafts in cytokine receptor signaling was also proposed. High-affinity IL-2 receptors were specifically found in detergent-resistant membrane complexes [79]. Electron microscopy studies supported the notion that IL-2Rα was partially confined to lipid rafts in a cholesterol-dependent manner. Although no functional studies have been published to date, the confinement of IL-2α receptor to lipid rafts suggest a mechanism whereby the less abundant β and γ chains of the IL-2 receptor may be recruited to form the fully functional IL-2R$\alpha\beta\gamma$ heterotrimer into lipid rafts.

Although further studies are warranted to identify the mechanism(s) by which GCs affect signal transduction in lymphoid cells, the available evidence suggest that GCs affect a membrane-proximal step of the signaling cascade of lymphocyte receptors whose function requires adequate membrane compartmentalization. Based on the

current literature, three possible mechanisms can be invoked, including (i) modification of the membrane-associated cholesterol content. This hypothesis is supported by numerous reports indicating that membrane cholesterol depletion causes impaired signaling [71, 72]; (ii) altered phospholipid composition [81]. Lipid rafts include predominantly saturated fatty acyl moieties. Addition of polyunsaturated fatty acids to T cell cultures leads to the selective modification of the plasma membrane cytoplasmic layer, resulting in marked displacement of Src family kinases (Lck, Fyn) from the lipid rafts and reduced TCR signaling [81]. Analysis of lipid composition in this model suggested that enhancing the degree of unsaturation within the GEMs could alter the inner lipid layer and as a consequence exclude several signaling proteins from these domains; or (iii) inhibition of protein acylation. Addition of lipid moieties to signaling molecules appears to be required for correct intracellular localization to the plasma membrane and to detergent-resistant GEMs. Mutational analyses have shown that proteins lacking acylation sites are excluded from the GEMs in transfected T cells and are unable to transduce signals issued from the TCR [82–85].

Although the effects of GCs on both lipid metabolism and the immune response have been well documented, no direct link between these two physiological responses has been considered so far. Based on the observations reviewed herein, it is tempting to speculate that GCs may affect the signaling properties of lymphoid cells through a transient modification of lipid metabolism.

Possible in vivo relevance

Numerous studies have suggested that the TCR does not act as a simple on/off switch, but rather is able to translate subtle changes in its ligand (antigen-MHC complexes) into unique signaling events leading to different phenotypic outcomes [48, 86]. In particular, T cells undergo a complex and ordered program of phenotypic changes during thymic selection, a process whereby autoreactive thymocytes are induced to die (negative selection), whereas thymocytes displaying self-MHC restricted potential are allowed to differentiate into mature T cells (positive selection) [48, 87, 88]. A currently favored model postulates that the "strength" of TCR signaling plays a crucial role during T cell differentiation in the thymus [48, 89]. Stimulation of developing thymocytes with a strong agonist peptide causes death by apoptosis (negative selection), whereas cells interacting with a low avidity ligand are induce to survive and differentiate into mature T lymphocytes (positive selection) (see fig. 1). In most models studied to date, the intensity of the signals issued by the TCR appears to be determined

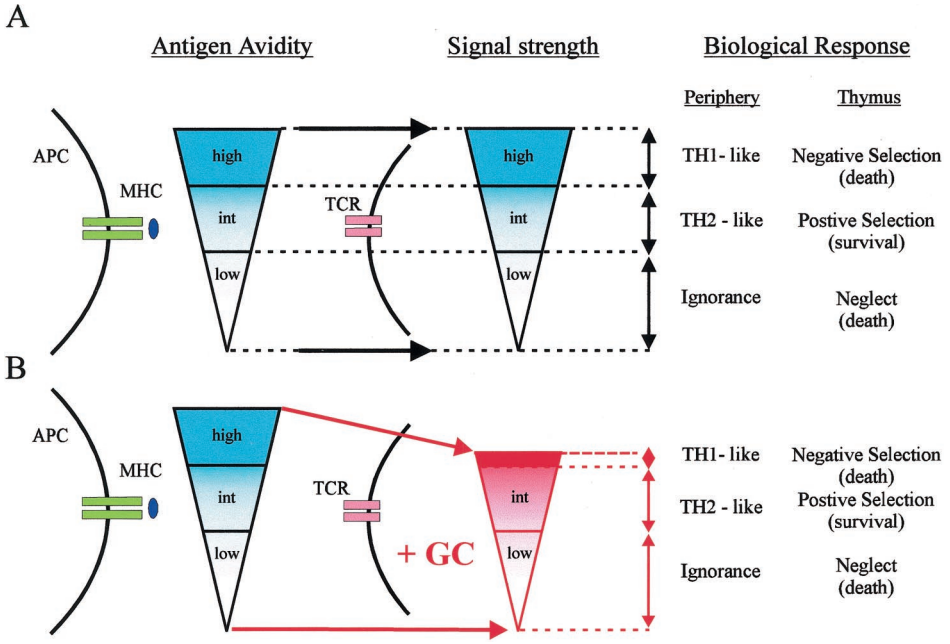

Figure 1. Ag/MHC complexes can display a wide range of affinities for a given TCR. The TCR translates these variable stimuli (antigen avidity) into graded intracellular signals displaying variable strength. Although the steps at which graded signals are converted into an all-or-none biological response remain poorly identified, numerous observations indicate that the degree of receptor activation is an important determinant of cell fate both in the periphery (Th1 vs. Th2 development) and in the thymus (positive vs. negative selection) (panel *A*). Glucocorticoids (GC) appear to affect the signaling capacities of the TCR without interfering with antigen avidity (panel *B*). High-avidity interactions are translated into intermediate signals in the presence of GC, thus favoring the development of Th2 responses in the periphery and the survival of developing thymocytes displaying sufficient self-reactivity.

by the overall avidity ("strength") of the T cell/antigen-presenting cell interaction, which is influenced by the affinity of the antigenic ligand for the TCR [48, 90]. The observation that GCs inhibit TCR signal transduction at an early step provides a sound biochemical basis for explaining the role of GCs in thymic development. By downregulating TCR signals, GCs may convert "strong" signals (normally causing negative selection) into a positively selecting, weaker signal. By lowering TCR sensitivity, GCs may shift the range of positive selected TCRs towards a higher self-reactivity, possibly ensuring that T cells surviving positive selection display a sufficient reactivity to self-MHC in the periphery. Notably, a weak level of self-reactivity is thought to be required for long-term survival of mature T lymphocytes in the periphery [91].

Signal strength has also been proposed to regulate the development of $CD4^+$ T helper cells in the periphery [37, 92]. In particular, "weak" signals (such as low dose or low-affinity antigens) are known to favor the differentiation of naive T cells into Th2-like cells, whereas priming of naive T cells with optimal doses of antigen will induce Th1 development [37, 93]. GCs may therefore modulate T helper responses in vivo by reducing TCR signal strength in response to antigen (fig. 1).

These observations suggest that by modulating TCR and cytokine receptor signaling properties, endogenous glucocorticoids produced during acute infection may favor the development of Th2-like cells with antiinflammatory properties.

Concluding remarks

GCs are known to affect numerous cellular responses in vivo. The precise mechanisms by which these compounds affect immune responses are difficult to delineate, as they probably rely on a multiplicity of cellular and molecular targets. The ability of GCs to affect an early step of the transmembrane signaling cascade in lymphoid cells has been largely unappreciated. This novel mechanism of immunoregulation requires long-term exposure of cells to GCs and de novo protein synthesis and may therefore have been overlooked for technical reasons in several in vitro models (see for example [94]). Better understanding of the effect of GCs on in vivo immune responses will require the identification of all potential mechanisms by which these natural or synthetic compounds affect lymphocyte responses. This knowledge will probably aid the development of better pharmacological strategies to downregulate unwanted in vivo immune responses such as inflammation and autoimmune diseases.

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