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Role of the Alternatively Spliced Glucocorticoid Receptor Isoform GRβ in Steroid Responsiveness and Glaucoma

Ankur Jain,[1,2] Robert J. Wordinger,[1,2] Thomas Yorio,[2,3] and Abbot F. Clark[1,2]

Abstract

Glucocorticoid (GC)-induced ocular hypertension (OHT) is a serious side effect of GC therapy in susceptible individuals. This OHT is due to increased aqueous humor (AH) outflow resistance in the trabecular meshwork (TM) caused by GC-mediated changes in TM structure and function. GCs may also play a role in the development of primary open-angle glaucoma (POAG). Elevated cortisol levels in the AH or enhanced GC sensitivity may be one of the reasons for elevated intraocular pressure in POAG patients. The GC OHT responder population is at greater risk of developing POAG compared with non-responders. We recently have gained insight into the molecular mechanisms responsible for this differential GC responsiveness, which is attributed to differences in GC receptor isoform expression in the TM. This article summarizes current knowledge on alternative GC receptor splicing to generate GC receptor alpha (GRα) and GRβ and their roles in the regulation of GC responsiveness in normal and glaucoma TM.

Introduction

The glucocorticoid receptor (GR) is a ligand-activated transcription factor that mediates numerous physiological functions and is the target for anti-inflammatory and immunosuppressive glucocorticoids (GCs). GCs are a group of natural (cortisol) or synthetic [dexamethasone (DEX), prednisolone] ligands that maintain normal metabolism, homeostasis, and immune regulation. Many of these properties of GCs have been exploited for anti-inflammatory, anti-allergic, and immunosuppressive uses. However, prolonged exposure to GCs can lead to a number of serious systemic and local adverse side effects. These adverse effects of GCs are dependent on GC potency, dosage form, pharmacokinetics, and route of administration. The systemic side effects include hyperglycemia, osteoporosis, immunodeficiency, and altered protein and lipid metabolism.

Ocular side effects of GCs include cataract and ocular hypertension (OHT) that can lead to glaucoma. Like primary open-angle glaucoma (POAG), GC-induced OHT is due to impaired aqueous humor (AH) outflow in the trabecular meshwork (TM). GCs have diverse effects on TM cells.[1] Experimentally, *ex vivo* studies using human and bovine eye anterior segments[2,3] and *in vivo* work in multiple species[1] prove the direct role of GCs in increased AH outflow resistance that is similar to glaucoma pathology in the TM. GCs increase extracellular matrix (ECM) proteins[4–7] decrease matrix metalloproteinase activity[8,9] and increase expression of TIMP1,[10] resulting in the deposition of ECM in the TM.[11] GCs reduce phagocytic activity of TM cells[12,13] that may further lead to increased outflow resistance and elevated intraocular pressure (IOP). In addition, GCs reorganize the TM cytoskeleton that affects TM cell migration, proliferation, and function,[14,15] and most likely also TM cell contractibility. GCs also increase the expression of myocilin, the first identified glaucoma gene,[16,17] but it is currently not known whether this plays any role in GC-induced OHT. Elevated levels of cortisol in the plasma[18–20] and AH[19] of POAG patients and their interactions with other pathways[21–23] may worsen the outcome of their disease.

Not all individuals equally respond to GC therapy, and there are clear cases of GC resistance and enhanced GC responsiveness.[24–31] There also are individual differences in GC responsiveness to the ocular side effect of GC-induced OHT. Normal individuals who are ''steroid responders'' develop elevated IOP (>6 mm Hg) following topical administration of GCs 3–4 times a day for 4–6 weeks.[11,32] Approximately 40% of the normal population are considered to be ''steroid responders.'' In contrast, almost all POAG patients are steroid responders.[32] Interestingly, non-glaucomatous steroid responders are at higher risk for developing POAG compared to non-glaucomatous non-responders.[33,34] One main reason for differential responsiveness to GCs at the

[1]Department of Cell Biology and Immunology, [2]North Texas Eye Research Institute, and [3]Department of Pharmacology and Neurosciences, University of North Texas Health Science Center, Fort Worth, Texas.

molecular level has been attributed to the relative expression levels of the 2 alternatively spliced GR isoforms GRα and GRβ.[24–26,28,30,35,36]

Regulation of GC Activity

There are a number of mechanisms that regulate GC activity (Table 1). GRα is the biological receptor for GCs and acts as a ligand-activated transcription factor. GRα is a >90 kDa protein that normally resides in cytoplasm. Binding to its highly lipophilic ligand (GC) causes a conformational change and the release of accessory proteins. Ligand-bound GRα is recognized by importins and translocated to nucleus (Fig. 1). Glucocorticoid response elements (GREs) on GC regulated genes facilitate the homodimerization of the GC-bound GR complex. This interaction between ligand-bound GR dimer and GREs drives transcription of various genes (transactivation). Binding of GRα homodimers to negative GREs can suppress gene transcription. Activated GRα can also alter gene expression in a GRE-independent manner by directly binding to and inhibiting other transcription factors such as AP-1 and NFkB in a process known as transrepression.[21–23] Activated GRα can also form heterodimers with other steroid receptors such as estrogen,[37] mineralocorticoid,[38] or androgen receptors.[39] Depending on which accessory proteins (coactivator/corepressor/HDAC) become associated with this complex, there is either inhibition or activation of GRE-mediated transcription. Similarly, the receptor heterodimers can bind to other responsive sequences on DNA such as estrogen response elements and depending upon the other factors recruited, transcription is either activated (transactivation) or repressed (transrepression). It was initially proposed that the major anti-inflammatory actions of GCs were mediated via transrepression, but this hypothesis has been recently challenged.[40] In addition to regulated interaction with other transcription factors, GR activity can also be regulated by transport into and out of the nucleus (Fig. 1). GCs can also trigger rapid signaling cascade independent of direct transcriptional or genomic changes.[41–43] Examples include effects on mitogen-associated protein kinases[44–52] and protein kinase C.[53–55]

Table 1. Mechanisms Regulating Glucocorticoid Activity in Trabecular Meshwork Cells

Mechanism	*References*
Potency of GC ligand	1,11
Alternative splicing regulating levels of GRα and GRβ	78,83
[Multiple translation initiation sites on GR mRNA]	58
[Factors affecting stability of GRα and GRβ mRNA]	
Nuclear import of GRα and/or GRβ	62,65
[Nuclear export of GRα and/or GRβ]	
Proteosome degradation of GRα and/or GRβ	62
[Heterodimerization with other steroid receptors]	37–39
[Presence of tissue-specific enhancers and/or repressors]	

"[]" indicates mechanisms shown in other cell types.
GC, glucocorticoid; GR, glucocorticoid receptor.

GC Receptor Beta

GR activity is also regulated by the different ratios of the alternatively spliced GRα and GRβ isoforms (Fig. 2). The GR gene *NR3C1* pre-mRNA (hnRNA) consists of 9 exons, and the last exon consists of 2 exons, 9α and 9β, separated by a short intronic sequence. Alternative splicing generates 2 isoforms: one incorporates exon 9α to form GRα and the other instead splices in exon 9β giving rise to GRβ.[56] Since the exon 9 encodes the ligand-binding (LB) domain (LBD), the resultant 2 isoforms greatly differ in their LB properties. GRα is a longer isoform with 777 amino acids and a fully functional LBD, whereas GRβ is a shorter version with 742 amino acids that loses its LBD and is thus unable to bind GCs.[56] The GRβ isoform is still capable of forming heterodimers with GRα, but the complex has substantially diminished transcriptional activity compared with the GRα-GRα homodimer. In this way, GRβ acts as a dominant negative inhibitor of GRα transcriptional activities and provides enhanced resistance to the biological and pharmacological effects of GCs. There is evidence that GRβ does possess transcriptional activity in stably transfected HeLa cells, but this is mainly due to interaction with other transcription factors or ligand-independent transactivation via the DNA-binding domain.[57] High GRβ levels are associated with GC-resistant asthma[24] and GC-resistant rheumatoid arthritis.[27] Both GRα and GRβ are expressed in the TM.[30,31] GRβ levels were found to be lower in TM cells isolated from glaucoma patients' eyes compared with TM cells derived from normal individuals.[30] Lower GRβ expression in the TM of glaucoma patients or steroid responders makes these cells more susceptible to GCs, leading to ECM buildup and cytoskeletal changes in the TM either by endogenous cortisol (such as found to be higher in AH of glaucoma patients) or by the administration of exogenous GCs (like DEX). This may explain the elevated IOP in glaucoma or the inherited tendency to elevate IOP in steroid responders. Adding further complexity to alternative splicing of the GR, multiple additional GRα and GRβ isoforms can be formed post-transcriptionally as the result of alternate translation initiation sites that can also regulate GC activity.[58]

The GRβ isoform has been conserved evolutionarily and can be traced back to zebrafish, suggesting its importance in regulation of GC activity. Although the patterns of splicing events differ among species, the ultimate properties and functions of GRβ remain the same by inhibition of GRα activities. In zebrafish, the splicing occurs at exon 8 of the GR gene instead of exon 9 of human GR gene.[59] The mouse GRβ (mGRβ) arises from alternative splicing utilizing intron 8 rather than exon 9 as in humans.[60] Similar intron inclusion occurs in generation of rat GRβ.[61] These GRβ proteins do not possess LB properties, show similar subcellular localization and expression levels, and act as dominant negative inhibitors of GRα activities in all these species.[59,60,61] Therefore, the GRβ isoform is invaluable in the study of steroid responsiveness. We have recently reported differential DEX responsiveness in an *ex vivo* perfusion culture model of bovine eyes anterior segments.[3] We are now in the process of determining whether altered

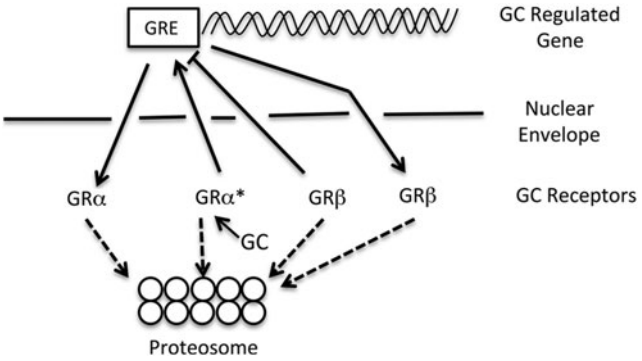

FIG. 1. Nuclear import and export of glucocorticoid receptor alpha (GRα) and GRβ and proteosomal degradation regulate glucocorticoid (GC) activities. Both GRα and GRβ proteins reside within the cytoplasm. Upon binding glucocorticoids (GC), GRα becomes activated (GRα*) and is translocated into the nucleus through the nuclear pore complex. GRα* then dimerizes and binds to glucocorticoid response elements (GRE) on GC-regulated genes to increase or decrease gene transcription. GRβ is translocated into the nucleus in a ligand-independent manner where it then acts as a dominant negative regulator by blocking GRα* activity. The levels of both GRα and GRβ can also be regulated by degradation of these proteins in the proteasome complex.

expression of bovine GRβ is responsible for these differences in DEX-induced OHT.

Regulated Nuclear Transport

Differences in the levels of GRβ can determine the steroid responsiveness among the individuals. In addition, nuclear import and export of the 2 GRα and GRβ isoforms can also regulate the GC activity (Fig. 1). The heat-shock protein 90 (HSP90), a molecular chaperone, is involved in nuclear transport of GRα and GRβ as shown by co-immunoprecipitation and transfection experiments.[62] Once in nucleus, GRβ inhibits the GRα transcriptional activity and provides GC resistance. Treatment with 17-AAG, an inhibitor of HSP90 chaperone activity, blocks the nuclear transport of GRβ and facilitates its degradation.[62] Therefore, proteosomal degradation of GRα or GRβ would alter the GRα/GRβ ratio and also regulate GC responsiveness in TM cells.[62] The FK-506 binding immunophilin FKBP51 has been implicated in GC resistance in primates.[63,64] In cultured human TM cells, FKBP51 maintains the constitutive GC-independent transport of GRα and GRβ from cytoplasm to nucleus, whereas FKBP52 is involved in nuclear transport of the GC-bound activated GRα, but FKBP52 is not involved in GRβ translocation.[65] FK506 facilitated FKBP51-mediated nuclear transport of GRβ and significantly reduced DEX-mediated GRE-luciferase activity in normal TM (NTM) cells, but had little effect on GRβ translocation in glaucoma glaucomatous TM (GTM) cells.[65] Indeed, FK506 potentiated DEX-mediated GRE-luciferase activity in GTM cells. These differential responses to FK506 may be explained by differences in cellular levels and/or activities of FKBP51 and FKBP52.

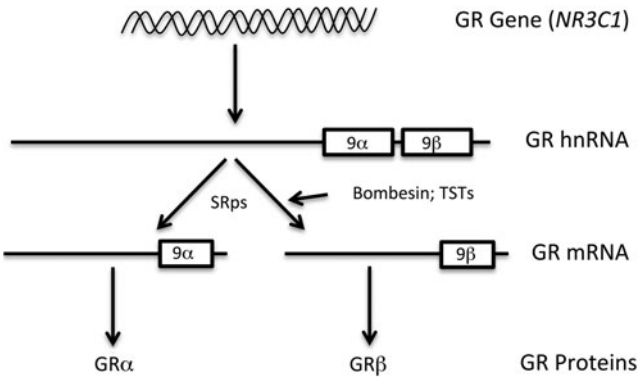

FIG. 2. Alternative splicing of the human GR. The GR gene (*NR3C1*) contains terminal exons 9α and 9β that are alternatively spliced from the primary hnRNA transcript to generate GRα and/or GRβ mRNAs. SRps 20, 30, and 40 in the spliceosome complex are involved in this differential splicing. Exogeneous compounds [bombesin or thailanstatins (TSTs)] also regulate this alternative splicing. The GRα and GRβ mRNAs are translated to form the ligand (GC) binding GRα isoform and the dominant negative regulator isoform GRβ.

Regulation of GR Splicing

Alternative mRNA splicing occurs for the majority of protein-encoding genes; more than 95% of transcriptome undergoes alternative splicing to generate diversity of mRNAs and proteins.[66] This process is tightly regulated inside cells. Any disruption in either cis-acting sequences on pre-mRNA or in activities/levels of trans-acting elements can affect this process, causing diseases. Alternative splicing is carried out by a specialized assembly of proteins and RNA called the spliceosome, which consists of 5 small nuclear ribonucleoproteins and different serine-arginine (SR) proteins (SRps).[67] RNA–RNA, RNA–protein, and protein–protein interactions ensure inclusion of the right number and order of exons in the final mRNA product.[68] In addition to 3 core sequences (i.e., 5′ splice site, 3′ splice site, and the branch sequence), there are additional sequences located within introns or exons or intron–exon junctions that recruit SRps.[69–71] Each SRp has a signature RNA recognition motif, and their levels or activities can modulate splice site recognition to promote alternative splicing. A critical balance of SRps and their antagonistic regulators is necessary for exon inclusion in mRNA transcripts.[72] These interactive SRps are also involved in mRNA nuclear export and translation, further elucidating their importance inside cells.[73–77]

In fact, SRps are master regulators controlling alternative mRNA splicing of GR (Fig. 2) and other genes in the TM[78] and other cell types.[79,80] Transient transfection experiments showed that SRp20 favors more GRα splicing, whereas SRp30c and SRp40 overexpression increases GRβ levels in TM cells.[78] The increased GRβ levels by overexpressing SRp30c or SRp40 are associated with decreased DEX activity in TM cells.[78] SRp20 overexpression increases GC responsiveness in NTM cells (which are low GC responder cells), whereas SRp30c and SRp40 overexpression decreases GCs responsiveness in GTM cells (which have greater GC responsiveness). There are differences in predicted binding sites for SRps on exon 9 of the GR gene, with more SRp20 sites on exon 9β and more SRp40 sites on exon 9α.[78] Also, SRp40 mRNA expression was lower in GTM

cell strains compared with NTM cell strains, which correlated to lower GRβ expression in GTM cell strains.[78] It would be interesting to determine what other TM cell genes SRp40 regulates in terms of alternative splicing and whether these genes are involved in glaucoma pathophysiology. Single-nucleotide polymorphism genotyping of DNA samples from normal controls, POAG patients, and steroid responders did not show any significant allele frequency differences for SRp20, SRp30c, or SRp40 between cohorts.[81] This suggests it could be the relative levels or activities of SRps that may determine GC responsiveness in these groups.

Peptides such as bombesin[78,79] and other chemical modulators of alternative splicing have further illustrated a role for SRps in determining GC responsiveness in TM cells. Thailanstatins (TSTs) are a new class of microbial-derived compounds that are very potent regulators of alternative mRNA splicing.[82] Bombesin and 3 TSTs (TST-A, TST-B, and TST-C) increase GRβ levels in TM cells and decrease GC responsiveness assessed by DEX induction of ECM (fibronectin) production, myocilin secretion, and GRE-luciferase reporter activity.[78,83] Currently, we are studying the TST mechanism of action and performing translational studies in *ex vivo* and *in vivo* models of steroid responsiveness to further clarify this mechanism. We will determine the effects of specific spliceosome modulators on a broad range of SRps. RNA interference-mediated knock down studies will also help identify what other SRps (in addition to SRp20, 30c, and 40) are involved in GR splicing. It is also possible that these compounds affect mRNA stability or the rate of mRNA transcription rather than direct interaction with the spliceosome.

Future Directions

The process of GR alternative splicing could possibly be exploited for therapeutic intervention. Manipulation of the alternative splicing to generate more GRβ in the TM would diminish endogenous cortisol activity in POAG patients and prevent GC-induced OHT in steroid responders. Another potential therapeutic approach to treat GC-induced OHT and glaucoma would be to use gene therapy to selectively overexpress GRβ in the TM. Several viral expression vectors, including adenovirus Ad5 and lentiviruses, have selective tropism for the TM,[84–87] which would prevent GC-induced biochemical, morphological, and physiological changes in TM but still allow the use of GCs for treating inflammatory conditions in steroid responders, including POAG patients. Bombesin and spliceosome modulators like TSTs have shown great promise *in vitro* in increasing GRβ in TM cell strains.[78,83] It would be worth testing these agents in *ex vivo* and *in vivo* models of GC-induced OHT. GC-induced OHT occurs in multiple species including non-human primates,[88] rabbits,[89] cats,[90] cows,[91] sheep,[92] rats,[93] and mice.[94] However, the trabecular outflow pathway in mice[95,96] and rats is more similar to primates, compared with these other species.

The present findings reinforce the need for appropriate steroid-induced glaucoma models. *Ex vivo* models of DEX-induced OHT involving perfusion of eye anterior segments provide fast and reliable tools to test these alternative splicing promoting compounds and determine their molecular mechanisms of action. We have demonstrated that DEX-induced OHT occurs in perfusion organ culture models of human eyes.[2] Mao et al. recently developed an *ex vivo* model of steroid-induced OHT using anterior segments of bovine eyes that is fast, reliable, and cost-effective.[3] GC-induced OHT in both these models occurs in ~40% of the DEX perfused eyes, which mimics the GC response seen clinically, and will allow us to determine the potential roles of GRβ in determining this dichotomous GC response on IOP. Cloning of the endogenous bovine GRβ will strengthen the effectiveness of the bovine model. Generation of GRβ knockout and GRβ transgenic mice will also help in understanding the physiological role of GRβ in GC-induced OHT. Overexpressing hGRβ or mGRβ in the mouse TM using viral expression vectors can be used to study the local effects on topically or systemically administered GCs. These proposed studies would help us better understand the physiological role of GR splicing and GRβ in IOP regulation. This would also help in understanding the role of GRβ in glaucoma etiology to aid in the discovery of novel therapeutic targets and strategies.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Dr. Abbot F. Clark
North Texas Eye Research Institute
University of North Texas Health Science Center
CBH-441
3500 Camp Bowie Boulevard
Fort Worth, TX 76107

E-mail: abe.clark@unthsc.edu