Mechanisms Generating Diversity in Glucocorticoid Receptor Signaling

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Glucocorticoids regulate diverse biological processes throughout the body via the glucocorticoid receptor (GR). Ligand-bound GR translocates into the nucleus and can elicit changes in gene expression by direct contact with the DNA or by protein–protein interactions with other transcription factors. The GR can also mediate rapid nongenomic signaling events initiated in the cytoplasm. In this chapter, we review the biological and physiological implications of glucocorticoids, the GR, and many of the signal transduction mechanisms that mediate their action.

Key words: **glucocorticoids; signaling; glucocorticoid receptor**

Introduction

Essential to human life, glucocorticoids are steroid hormones that allow us to cope with a variety of environmental and physiological stresses. Glucocorticoids are produced and released by the adrenal cortex under the control of the hypothalamic-pituitary-adrenal axis (HPA). When the body perceives stress, the hypothalamus releases corticotropin-releasing hormone (CRH) that stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. The adrenal cortex, in turn, synthesizes glucocorticoids in response to ACTH. Thus, glucocorticoids are ultimately part of an adaptive response that seeks to maintain homeostasis during stressful situations. Indeed, the most notable physiological reactions to glucocorticoids, increased glucose production and immunosuppression, are responses to some of the most common insults the human body encounters, namely low blood glucose and inflammation, respectively.[1,2]

Once produced by the adrenal cortex, glucocorticoids are released into the bloodstream and bind corticosteroid-binding globulins to be distributed throughout the body. Due to their hydrophobic nature, glucocorticoids readily diffuse from the circulation into organs, tissues, and cells. Intracellularly, glucocorticoids interact with the ubiquitously expressed glucocorticoid receptor (GR) to orchestrate a vast array of responses at the cellular level that culminate in changes in metabolism (carbohydrate, protein, and lipid), immune system function, vascular tone, bone mineralization, and central nervous system function, among others.[1]

Dysregulation of glucocorticoid production, as seen in Cushing's or Addison's disease, can lead to severe complications and ultimately death. Cushing's disease is characterized by overproduction of glucocorticoids in the adrenal cortex as a result of high ACTH levels because of a pituitary gland tumor. The disease is characterized by weight gain, increased fat mass, hyperglycemia, reduced muscle and bone mass, immunosuppression, and water retention. Untreated, these symptoms can lead to diabetes, hypertension, heart failure, edema, and infection. Surgical removal of the pituitary

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tumor is the only successful treatment for Cushing's disease.[3]

Addison's disease, in contrast, is characterized by decreased production of glucocorticoids, leading to hypocortisolism. It is generally caused by developmental defects or trauma to the adrenal cortex that results in the deficient production and secretion of glucocorticoids and mineralocorticoids. Symptoms include weight loss, hypoglycemia, and alteration of sodium and potassium levels. Addison's disease can be successfully treated with glucocorticoid- and mineralocorticoid-replacement therapy.[4]

Largely due to their powerful immunosuppressant activity, many synthetic glucocorticoids have been developed and are currently in therapeutic use. These compounds are some of the most commonly prescribed drugs in the world today. They are used in the treatment of many hematological cancers as well as in asthma, allergic rhinitis, ulcerative colitis, rheumatoid arthritis, eczema, and psychological disorders of the brain. Unfortunately, due to their broad range of physiological actions, long-term use of glucocorticoids is invariably accompanied by adverse side effects that bear close resemblance to Cushing's disease. Currently, a considerable amount of research seeks to generate synthetic glucocorticoids with more targeted actions and reduced side effects.

The Glucocorticoid Receptor Gene

The GR is a steroid hormone receptor that belongs to the nuclear receptor superfamily of transcription factors. It is related to other steroid receptors, such as the mineralocorticoid, androgen, estrogens, progesterone, vitamin D, and retinoic acid receptors.[5] These steroid receptors are evolutionarily conserved in mammals and believed to have originated by multiple gene duplications from a common ancestor in a process that began 400 million years ago.[6]

The GR is essential for life, as illustrated by multiple GR knockout mouse models.[7–9] Whole body deletion of exon 2 of the mouse GR gene results in severe developmental abnormalities in the lung that lead to death within hours of birth.[7] More recently, the GR has also been disrupted in a tissue-specific manner by deletion of either exon 2 or exon 3. In the liver, GR has been shown to be responsible for gluconeogenic metabolism, as well as insulin-like growth factor (IGF)-1 production required for postnatal growth.[10,11] Central nervous system–deficient GR mice exhibit dysregulation of the HPA axis and develop a number of physiological and behavioral abnormalities that mimic depressive disorders.[12,13] Furthermore, deletion of GR in T cells seems to be dispensable for T cell development but results in increased mortality upon immune activation due to dysregulation of several cytokines.[8] Similarly, macrophage GR-deficient mice also exhibit severe mortality caused by increased cytokine production in response to activation of inflammatory signaling.[9] Finally, osteoclast GR-null mice develop normally and are protected from glucocorticoid-suppressed bone formation.[14]

The human GR (hGR) gene is located on chromosome 5 (region 5q31p) and is composed of 9 exons (Fig. 1A).[15,16] In the mature mRNA, exon 1 represents the 5′-untranslated region, while exons 2–9 code for the protein and the 3′-untranslated regions (Fig. 1B). Exon 1 is composed of several independent variants termed 1A-1J, each of them containing its own transcriptional start sites and unique promoters.[17,18] Interestingly, none of these promoters contain classical TATA- or CCAAT-boxes.[15,17–19] The varieties of promoters, transcription factor–binding sites, and transcriptional start sites are thought to account for the differences in gene expression of hGR throughout the body. Similar to exon 1, exon 9 can be alternatively spliced to produce the two most widely known isoforms: hGRα and hGRβ (Fig. 1B).[16]

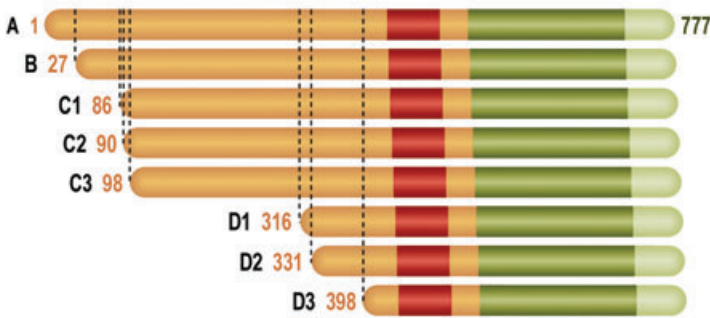Isoforms hGRα and hGRβ are identical up to amino acid residue 727, but differ on their
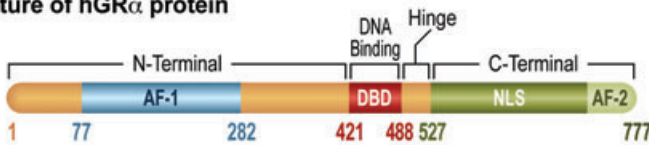
Figure 1. The hGR gene codes for multiple isoforms. (**A**) The hGR gene is located in chromosome 5 (region 5q31p) and is composed of nine exons. Exons 1 and 9 contain multiple variants. (**B**) Alternative splicing of exon 9 gives rise to the mRNA messages of the two most characterized hGR isoforms—hGRα and hGRβ. (**C**) Alternative initiation sites can give rise to seven more isoforms from each mRNA message. (**D**) The domain structure of full-length hGRα, or hGRα-A. It is composed of an N-terminal region, a DNA-binding domain, a hinge region, and a C-terminal region. The N-terminal region contains an AF-1 domain able to interact with the basal transcriptional machinery. The DNA-binding domain contains two zinc fingers involved in dimerization and interacts with the DNA. The hinge region contains NLS. The C-terminal domain contains the ligand-binding motif, an NLS, and an AF-2 involved in interactions with various transcriptional regulatory factors. (**E**) Phosphorylation of serines 203, 211, 226, and ubiquitination of lysine 426 are known post-translational modifications of hGRα.

carboxy termini. The predominant isoform is hGRα and is composed of 777 amino acid residues. It is capable of binding endogenous glucocorticoids and mediating a variety of signaling mechanisms. In contrast, hGRβ is only 742 amino acids long and generally expressed at much lower levels than hGRα.[20] While the physiological relevance of hGRβ is unclear, it is believed to act as a dominant negative regulator of hGRα.[21] Higher expression of hGRβ has been associated with cardiovascular disease, as well as glucocorticoid-resistant asthma, ulcerative colitis, and rheumatoid arthritis.[22] hGRβ is unable to bind any known glucocorticoids and reported to be transcriptionally inactive. Recent studies have shown that hGRβ can bind RU-486, a synthetic glucocorticoid antagonist. Moreover, the mere expression of hGRβ is sufficient to elicit changes in gene expression, and many of these changes largely disappear in the presence of RU-486.[23] Other splice variants have been identified and code for isoforms hGRγ, hGR-A, and hGR-P.[24–26] However, their expression level is low in comparison to hGRα, and their physiological role is unclear.

Additional GR isoforms are also generated by alternative translation initiation of the mature mRNA (Fig. 1C). These isoforms are created when the ribosome skips an upstream initiation codon, either by leaky scanning or ribosomal shunting, and starts translation from an alternative initiation site located further downstream in the mRNA message. The resulting protein isoforms exhibit progressively shorter N-terminal regions, but have identical amino acid sequences downstream of the alternative initiation sites. The hGRα and hGRβ mRNA transcripts can each give rise to eight translational isoforms, many of which exhibit different tissue-expression patterns and mediate unique transcriptional responses.[27,28]

Protein Structure

GRα is a modular protein and is comprised of three domains: the N-terminal transactivation domain, the central DNA-binding domain, and the C-terminal ligand-binding domain (Fig. 1D). The N-terminal domain contains the AF-1 (activation function) motif that can physically interact with the basal transcriptional machinery to mediate gene activation.[29] The DNA-binding domain harbors two zinc finger motifs and is required for receptor dimerization as well as interactions with the DNA.[30] The C-terminal domain contains the ligand-binding motif, a nuclear localization signal (NLS), and a second activation function motif (AF-2) involved in interactions with various transcriptional regulatory factors.[30] Crystal structures have revealed that the ligand-binding motif is a hydrophobic pocket created by numerous α-helices and β-sheets.[31] In addition to these three large domains, there is a small flexible portion of the protein called the hinge region. It is located between DNA-binding and the C-terminal domains and contains an additional NLS.[30]

The GRα protein can also be modified post-translationally by phosphorylation, sumoylation, and ubiquitination (Fig. 1E).[32] Phosphorylation of hGRα at serines 203, 211, and 226 leads to changes in its subcellular localization and ability to interact with other proteins, whereas ubiquitination of lysine 426 alters its cellular half-life.[33–35] The exact physiological relevance of these and other post-translational modifications of GRα remain to be established; however, it is widely believed that they can modify glucocorticoid signaling.

Based on the presence or absence of hormone ligand, intracellular GRα appears to exist in two conformations (Fig. 2). In the first, the receptor is ligand unbound and sequestered in the cytoplasm in a multimeric complex composed of hsp90, hsp56, hsp40, p23, Src, and others.[36,37] This complex keeps the receptor in a conformation poised to bind its ligand and conceals the NLSs. Ligand binding (glucocorticoids) induces a conformational change in GRα that leads to its dissociation from the multimeric complex and exposes its NLSs,
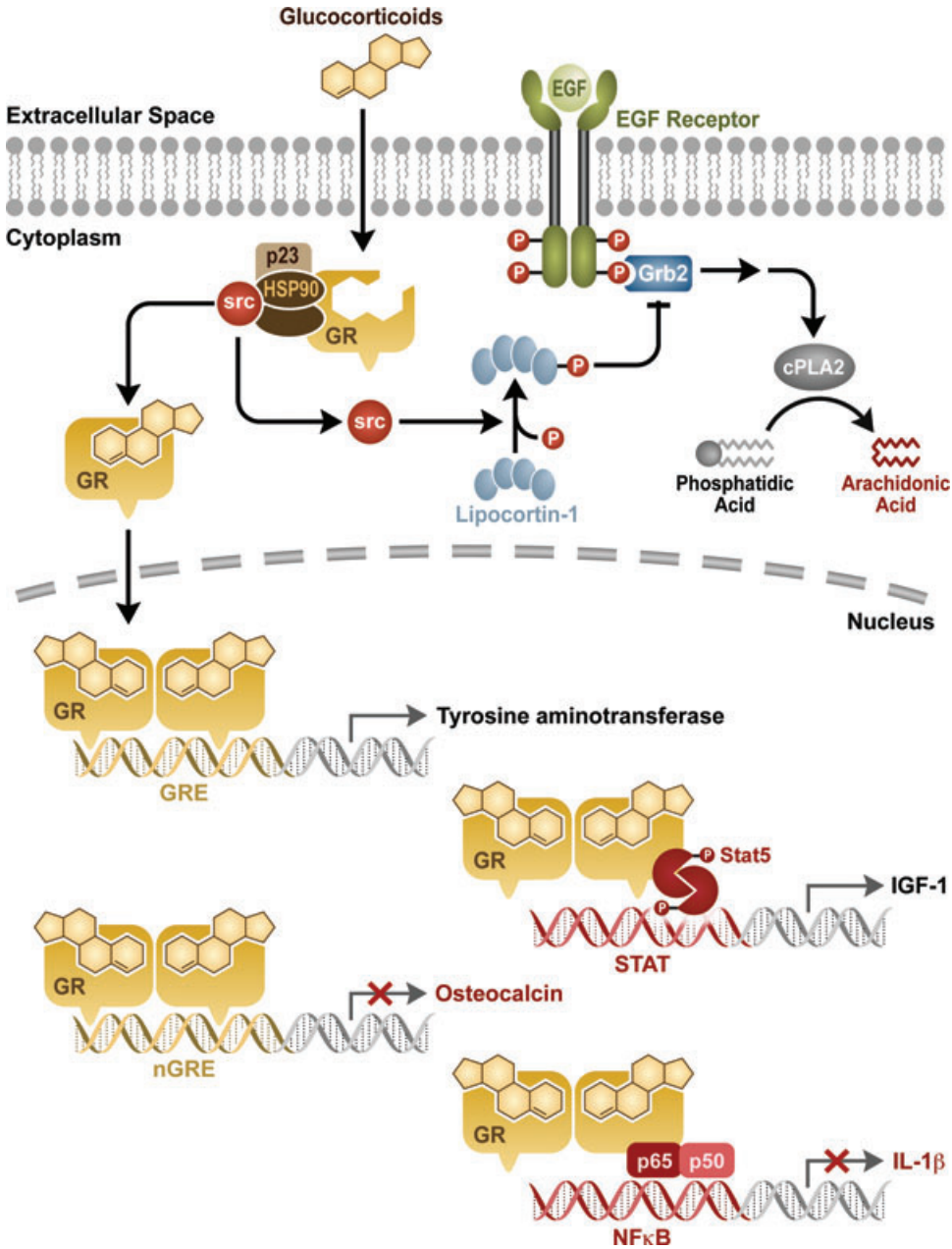
Figure 2. Glucocorticoid signaling results in changes in gene expression and rapid nongenomic events in the cytoplasm. Ligand binding to the GR leads to the dissociation of a cytoplasmic complex interacting with GR. Ligand-bound GR rapidly translocates into the nucleus, where it can activate gene expression by directly binding to GREs in the DNA, or associate with transcription factors, such as STAT5. GR can also promote gene silencing by directly interacting with nGREs in the DNA, or by protein–protein interactions with transcription factors, such as NFκB. In the cytosol, the dissociation of the protein complex interacting with GR leads to the release of src kinase, which phosphorylates lipocortin-1. Phosphorylated lipocortin-1 displaces Grb2 from the activated EGF receptor to inhibit the activation of cPLA$_2$ and the creation of arachidonic acid.

resulting in its rapid translocation into the nucleus.[36]

Gene Activation by Direct DNA Binding

In the nucleus, ligand-bound GRα dimerizes and binds to the DNA in a sequence-specific manner. The DNA sequences where GRα binds are called glucocorticoid response elements, or GREs, and are characterized by similarity to the 15-bp consensus sequence 5′-AGAACAnnnTGTTCT-3′ (Fig. 2). However, in numerous genes, GRE half-sites are sufficient to elicit GRα-mediated transcriptional changes.[38] Multiple GREs are usually distributed in the proximal promoters of target genes, but their numbers and location can vary substantially.[39,40] DNA binding by GRα induces a conformational change in the receptor that results in the physical association with a variety of coregulatory factors and their recruitment to the chromatin.[2]

Several mechanisms exist by which GRα and DNA interactions promote gene induction. First, if the GRE is in close proximity to the TATA-box, hGRα can recruit key components of the basal transcriptional machinery to the TATA-box, such as Transcription Factor IID, and thus directly promote gene activation.[41] If the GRE is located at a distance of the TATA element, hGRα can associate with coactivators that function as bridges to promote the recruitment of the basal transcriptional machinery.[42,43] Furthermore, GRα can also recruit chromatin-remodeling coactivators that alter the nucleosomal structure of the DNA and create a more favorable environment for gene expression. Some of these coactivators, such as CBP, p300, p/CAF, and SRC-1, are histone acetyl transferases (HATs), while others, such as SWI/SNF, are ATP-dependent chromatin remodeling factors.[44,45] Examples of genes positively regulated by GREs include tyrosine aminotransferase, alanine aminotrans-

ferase, and phosphoenolpyruvate carboxykinase, all involved in liver gluconeogenesis.

Gene Activation by Protein–Protein Interactions

GRα can also regulate gene activity independent of DNA binding via protein–protein interactions with other transcription factors (Fig. 2). GRα-dependent gene activation by this mechanism is best illustrated by Signal Transduction and Transcription proteins (STATs). STATs are transcription factors involved in the Janus kinase (JAK) signaling pathway. Activation of JAK signaling results in the phosphorylation and dimerization of STATs, leading to their translocation into the nucleus and subsequent interaction with their response elements in the DNA.[46] It has been shown that STAT-5 physically interacts with GRα. In this case, STAT-5 is directly associated with the DNA, while GRα is recruited to the chromatin without it directly interacting with the DNA. The GRα–STAT-5 association leads to activation of several genes, most notably IGF-1 in the liver that is required for postnatal growth.[11] GRα is also capable of interacting with STAT-3 and -6 to promote gene activation.[47,48]

Gene Repression by Direct DNA Binding

Direct DNA binding by GRα can also lead to repression of genes by interactions with negative GREs (nGREs) (Fig. 2). nGREs are similar to GREs and almost always are located in close proximity to DNA-binding sites for other transcription factors necessary for gene expression. As a result, hGRα–nGRE interactions lead to gene silencing by competing with, and displacing, other transcription factors from the DNA. The osteocalcin gene promoter, for instance, contains an nGRE that overlaps with its TATA box, and GRα association with this site

prevents access from the basal transcriptional machinery.[49] Similarly, the human FasL gene contains an nGRE adjacent to a nuclear factor-κ B (NFκB) site.[50] GRα–nGRE association in this site prevents NFκB binding and induces gene silencing. Despite the fact that nearly half of the genes regulated by glucocorticoid signaling are repressed, only a handful of them are known to be regulated by nGREs. These include pro-opiomelanocortin, CRH, prolactin, and neuronal serotonin receptor.

Gene Repression by Protein–Protein Interactions

Most of the genes repressed by GRα occur via protein–protein interactions independent of DNA binding by the receptor (Fig. 2). This mechanism of signaling has been best characterized for the transcription factors NFκB, activator protein-1 (AP-1), and Smad3. NFκB is a ubiquitous homo/heterodimer transcription factor most widely known for its role in inflammation that is composed of subunits from the Rel family of proteins (p50, p52, p65, RelB, and c-Rel). The p50/p65 heterodimer is the most common combination involved in transcriptional processes.[51] In cells, NFκB is kept in an inactive state in the cytoplasm by interactions with its inhibitor, IκB.[51] Several stimuli (usually inflammatory) result in the activation of IκB kinase that phosphorylates IκB to promote the dissociation of the IκB–NFκB complex.[51] Free NFκB translocates into the nucleus, where it binds to DNA regulatory elements and activates gene transcription.[51]

GRα can physically bind to p65 and repress the NFκB-mediated transcription in several ways. For example, the interaction of GRα with p65 can result in the sequestering of the NFκB complex, thereby preventing NFκB from reaching its DNA-binding site.[52] GRα can also interact with DNA-bound NFκB to inhibit the recruitment of the transcriptional machinery.[52] Furthermore, since GRα and NFκB require many of the same transcription activators, such as CBP or p300, it is believed that GRα can diminish the available pool of these factors, thereby negating their use by NFκB.[53] Finally, GRα can also suppress NFκB transactivation by interfering with the cellular machinery necessary for gene activation. In this regard, GRα has been reported to interfere with the NFκB-mediated phosphorylation of the RNA Polymerase II C-terminal tail, necessary for robust transcriptional activation.[54] GRα has also been shown to obstruct p65-mediated HAT activity while promoting the recruitment of histone deacetylase-2 to NFκB target genes.[55,56] Together, these effects increase chromatin tightening and result in decreased gene expression. Classical gene targets of NFκB that are repressed by GRα include many pro-inflammatory cytokines and their receptors, such as tumor necrosis factor α (TNFα), interleukin-1β, and granulocyte monocyte colony stimulating factor.

Similar to NFκB, GRα can bind and repress the transcriptional activity of AP-1. AP-1 is a homo/heterodimer transcription factor composed of Fos family (c-Fos, FosB, Fra1, and Fra2) or Jun family members (c-Jun, JunB, and JunD).[57] It binds to its DNA response element via a basic leucine zipper motif. A variety of stimuli can lead to phosphorylation of AP-1 complexes and greatly increase their transcriptional activity. GRα can physically interact with AP-1 and inhibit AP-1-mediated gene activation by employing similar mechanisms to the ones discussed for NFκB.[58] Genes repressed by GRα in an AP-1-dependent manner include collagenase, stromelysin, and other matrix metalloproteinases.

Inhibition of Smad signaling is yet another case where GRα can bind and repress the activity of a transcription factor. In this pathway, the activated tumor growth factor β (TGFβ) receptor phosphorylates Smad3 and promotes its association with Smad4. The Smad3–4 complex translocates into the nucleus and acts as a transcription factor by binding to DNA

regulatory elements. Physical interactions with GRα, however, greatly reduce the transcriptional activity of Smad3–4 complexes.[59] It is unclear how GRα inhibits Smad3-mediated gene activation, but mechanisms similar to ones already discussed are probably at play.[59,60] TGFβ–Smad signaling is important for cell differentiation, extracellular matrix production, as well as immune and inflammatory responses. Other examples of GRα-mediated gene repression by protein–protein interactions include transcription factors NF-AT and IRFs, both well known for their role in inflammation.[46,53,61]

Signaling by Translational Isoforms

As previously mentioned, alternative initiation sites in the mRNA transcript of GRα give rise to eight translational isoforms.[27] The classic GRα, with 777 amino acids, is actually isoform GRα-A. The other seven isoforms, in descending order of molecular size, are GRα-B, -C1, -C2, -C3, -D1, -D2, and -D3. All have identical DNA-binding and C-terminal ligand-binding domains but exhibit truncated N-termini compared to GRα-A. In the extreme case of GRα-D3, for instance, the N-terminal domain is almost completely missing.

Despite having similar ligand affinity, these translational isoforms elicit different gene expression changes. In particular, it was recently found that isoform GRα-C3 is more efficient at inducing proapoptotic genes in human osteosarcoma cell lines than the classical GRα-A. Conversely, isoform GRα-D3 exhibited a decreased capacity to generate the same transcriptional responses. The molecular mechanism of action seems to involve an enhancement in the recruitment of coactivators by GRα-C3, and the opposite occurs with isoform GRα-D3.[62] While the expression levels of these translational isoforms vary among tissues, it is still unknown what their physiological impact might be, or what factors determine their expression patterns.

Secondary Signaling Mechanisms

Primary GRα-mediated changes in gene expression initiate countless other signaling events. For instance, glucocorticoid signaling has been shown to alter the mRNA turnover of TNFα, an inflammatory cytokine. Here, GRα activation leads to the increased expression of tristetraprolin (TTP), a protein equipped with zinc finger domains capable of binding to mRNA molecules containing adenylate-uridylate-rich elements (AREs) located in the 3′ untranslated regions.[63] Association of TTP with AREs leads to the rapid decay of the target mRNA by recruitment of RNA degrading enzymes.[64] GRα-mediated gene induction of TTP increases the mRNA turnover of TNFα transcripts through AREs, and leads to a reduction in TNFα protein synthesis.[63] Since many other mRNA transcripts contain AREs, it is possible that glucocorticoid signaling regulates the expression of other genes in this manner.

Glucocorticoids can also elicit secondary responses by altering the components of other signaling pathways. For instance, primary GRα signaling induces the expression of the mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP1) gene. Increased expression of MKP1 leads to a decrease in MAPK signaling by dephosphorylating and inactivating the MAPK components JNK, ERK, and p38.[65] The MAPK pathway is involved in diverse cellular processes, such as apoptosis, mitosis, and differentiation.

A change in the expression level of a transcription factor is yet another mechanism by which primary GRα signaling can lead to secondary cellular events. Glucocorticoids, for instance, silence the expression of ATF4, a basic leucine zipper transcription factor required for the expression of genes involved in amino acid biosynthetic enzymes, amino acid transporters, and aminoacyl-tRNA synthetases.[66]

Therefore, primary GRα signaling facilitates the switch from amino acid and protein anabolism to catabolism through the downregulation of ATF4.[66] It is possible that many of the physiological actions ascribed to glucocorticoids are the result of secondary changes in gene expression mediated by transcription factors.

Nongenomic Signaling

In addition to the variety of genomic signaling mechanisms discussed, there is increasing evidence that glucocorticoids are capable of rapid signaling events independent of transcriptional changes. One of the most notable examples involves a reduction in the production of the pro-inflammatory molecule arachidonic acid (AA) by inhibition of the epidermal growth factor (EGF) signaling pathway (Fig. 2).[67] Here, Src kinase, part of the multimeric complex sequestering GRα, is released upon hormone binding and phosphorylates lipocortin-1.[67] Phosphorylated lipocortin-1, in turn, displaces the adaptor protein Grb2 from active EGF receptors. Reduced EGF signaling through Grb2 results in diminished activity of cytoplasmic phospholipase A_2 (cPLA$_2$), whose enzymatic byproduct is AA.[67] Another rapid, nontranscriptional event associated with glucocorticoids involves the phosphoinositide 3-kinases (PI3Ks) signaling pathway, leading to protective effects on the cardiovascular system and the brain. Physical interaction of GRα with p85α, the main regulatory subunit of PI3Ks, can lead to increased PI3Ks activity and downstream signaling.[68,69] Further research is necessary to determine the physiological relevance of these and other non-genomic signaling events mediated by GRα.

Summary

In this chapter, we have reviewed several mechanisms by which glucocorticoids operate and elicit signaling events via the GR. Glucocorticoids are steroid hormones released by the adrenal cortex under situations of environmental or physiological stress. In the cell, glucocorticoids bind to the GR and elicit the disassembly of a multimeric protein complex sequestering the GR in the cytoplasm. Ligand-bound GR translocates into the nucleus and alters gene expression via GREs or nGREs, both of which involve direct DNA binding. Alternatively, the GR also mediates transcriptional changes without direct DNA binding by physical interactions with transcriptional factors, such as STATs, NFκB, AP-1, and Smad3. Translational isoforms of the GR selectively regulate gene expression, and thus greatly increase GR signaling mechanisms. In addition, secondary signaling events of glucocorticoid action can affect mRNA turnover or alter the composition of other signaling cascades. Gene induction or repression of transcription factors by GR can further elicit secondary changes in gene expression. Finally, rapid nongenomic signaling events of glucocorticoid signaling involve the disassembly of the cytoplasmic complex sequestering GR, or cross-talk by protein–protein interactions with components of other signaling pathways. Clearly, the innumerable biological processes that glucocorticoid signaling influence are matched by an astonishing diversity of signaling mechanisms.

Conflicts of Interest

The authors declare no conflicts of interest.

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