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Commentary

Designer glucocorticoids

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Abstract

Glucocorticoids are the most effective anti-inflammatory agents known. However, the use of these powerful molecules is plagued by a host of serious, sometimes life-threatening side-effects. The search for new compounds that maintain the efficacy of the steroids without some of the side-effects has entered a new phase. New approaches are leading to novel kinds of steroidal and non-steroidal compounds with unique profiles that may represent the next generation of safer glucocorticoids.

Keywords: Selective glucocorticoid receptor modulators; Steroid receptor ligand; Non-steroidal anti-inflammatory agent

1. History of glucocorticoids

Early efforts to understand endocrine function focused on extracts derived from glands whose removal caused specific systemic pathologies in animals. The adrenal was one such gland. Organic extracts from this gland could be used to ameliorate the symptoms of Addison's disease (later discovered to be caused by the specific lack of the glucocorticoid hormone cortisol). Addison's patients exhibit hyperpigmentation of the skin, hypoglycemia, and salt craving, among other symptoms. These patients do not respond effectively to stress, creating the potential for an Addisonian crisis, a disease cascade that is potentially life-threatening. The majority of these symptoms can be eliminated with the injection of adrenal extracts.

The isolation of the active components from this gland by Tadeus Reichstein and Edward Kendall, and the subsequent use in patients with rheumatoid arthritis by Philip Hench, garnered all three the Nobel Prize in 1950. These agents were remarkably effective at inhibiting many forms of inflammation and were used at high doses over long periods of time, resulting in excellent efficacy. Unfortunately, early on, it was discovered that these compounds had a severely negative impact on patients to whom they were administered. Efforts from that point on have focused on finding molecules that have anti-inflammatory efficacy equal to that of the steroids, but with a reduction in side-effects. Progress has been made with several synthetic steroidal versions that exhibit increased receptor specificity and potency (dexamethasone) as well as versions for use in topical, inhaled, or other non-oral formulations. These, typically, are extremely potent steroids with high efficacy that are cleared rapidly by first-pass metabolism. This profile results in excellent efficacy at the point of application (e.g. lung and skin) but with reduced, although not absent, systemic exposure [1,2].

2. Glucocorticoid physiology

Corticosteroids produced in the adrenal gland undergo metabolism into two compounds (glucocorticoids and mineralocorticoids) with markedly different activities; glucocorticoids like cortisol have effects on carbohydrate, fat, and protein metabolism, and mineralocorticoids like aldosterone have effects on sodium levels by raising reabsorption in the kidney [3]. The responses to these hormones are mediated by two different intracellular receptors, which are capable of binding to and thereby being activated by these steroids, translocating to the nucleus, and regulating specific target genes. This signal transduction pathway is unique in that it utilizes a single protein that is both the proximal ligand receptor as well as

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Abbreviations: CRH, corticotropin-releasing hormone; CRF, corticotropin-releasing factor; ACTH, adrenocorticotropin hormone; HPA, hypothalamic-pituitary-adrenal; GVHD, graft-versus-host disease; PEPCK, phosphoenol pyruvate carboxy kinase; LBD, ligand-binding domain; GRIP-1, GR interacting protein 1; NFκB, nuclear factor-kappa B; AP-1, activator protein 1; PPAR, peroxisome proliferator activated receptor; PGC-1, PPARγ coactivator 1; HNF-4, hepatocyte nuclear factor 4.

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the transcription factor that interacts with DNA in the nucleus. The GR, in particular, is responsible for up-regulating and down-regulating a wide range of genes affecting a number of critical metabolic pathways. Glucocorticoids are commonly known as the stress hormones, and, under normal circumstances, they are crucial to the ability of the body to respond and adapt to stress. Stress causes signaling within the two key components of the stress response, CRH neurons found in the paraventricular nucleus of the hypothalamus and in other areas of the central nervous system as well as the noradrenergic neurons of the locus-ceruleus-norepinephrine system. Activation of these systems induces psychological, behavioral, and physical changes that provide an adaptive benefit in the short term. The HPA axis is on the receiving end of signals from the CRH neurons. This axis is the primary regulator of endogenous glucocorticoid production. CRF from CRH neurons acts on the anterior pituitary gland to cause the secretion of ACTH, which, in turn, induces corticosteroid production and release from the adrenal gland. Thus, in response to stress, the HPA axis is activated, and glucocorticoid levels in serum increase. The behavioral and psychological changes that occur when cortisol levels rise are indicative of a fight or flight response and include increases in alertness, mental acuity, pain tolerance, temperature, and euphoria coupled with decreases in sexual desire, reproductive behavior, and appetite [4]. The physical/physiological changes include increases in respiration rate, oxygenation, cardiovascular tone, blood flow, pressure, and heart rate. Accompanying these are adjustments in metabolism to provide glucose and energy by increasing gluconeogenesis in the liver and lipolysis in fat depots. Protein is broken down to provide substrates for gluconeogenesis. A number of peripheral systems unnecessary to the short-term stress response are inhibited. These suppressed functions include growth, reproduction, food absorption, and the immune response. Interestingly, the stress response also provides an autoregulatory mechanism to reduce the production of glucocorticoid after high levels have been achieved. This is accomplished by feedback of glucocorticoids onto the signaling pathways that induce glucocorticoid production. Glucocorticoids inhibit the expression of genes involved in activating the HPA axis, including corticotropin-releasing factor and ACTH, thereby regulating corticosteroid production from the adrenal gland [4].

There are several situations when this carefully balanced system can go awry. Chronic, uncontrolled stress leads to long-term activation of the HPA axis and sustained, high glucocorticoid levels. Pathological conditions in which activation of the HPA axis has been demonstrated include depression, obsessive—compulsive disorder, alcohol and drug abuse, and anorexia nervosa [4–7]. The action of glucocorticoids in response to chronic stress is decidedly not beneficial.

Imbalances in cortisol production can also occur in certain conditions that overproduce glucocorticoids. These patients present with a combination of symptoms grouped under the heading ‘Cushing’s syndrome’ [8]. The symptoms include central obesity, glucose intolerance, myopathy, and hypertension, among others. The pathophysiology of this condition is most often related to dysregulation of the system that produces glucocorticoids due to tumors or other endocrine problems. Certain adrenal tumors are known to secrete large quantities of cortisol and are not regulated by normal feedback mechanisms. Over-secretion of signaling molecules within the glucocorticoid production pathway, CRF or ACTH by the pituitary or non-pituitary cells, can also increase serum levels of cortisol substantially.

The last and perhaps the most common way in which imbalances in the stress response system can present themselves is when patients are administered exogenous glucocorticoids.

Glucocorticoids are extremely effective and frequently used therapeutic agents that are administered for a wide range of disorders. Supplemental glucocorticoids can replace cortisol absent in various adrenal insufficiency syndromes [3,9]. These compounds are also effective anti-inflammatory agents for many autoimmune and inflammatory disorders, such as rheumatoid arthritis and asthma. With rheumatoid arthritis, treatment with non-steroidal anti-inflammatory drugs (NSAIDs) provides significant benefit; however, the progression of the disease eventually demands the use of glucocorticoids. Steroids inhibit the signs and symptoms of the inflammation associated with rheumatoid arthritis, but fail to reverse any structural damage that has already occurred in the joint. Additionally, patients with asthma often use inhaled and oral steroids to control exacerbation of their condition. Immunosuppressive therapy for transplant rejection and autoimmune disorders often makes use of short-term, high-dose treatment with steroids followed by more protracted lower dose treatment to reduce the cell-mediated response to transplanted foreign tissue. Patients receiving bone marrow transplants occasionally develop acute GVHD. Glucocorticoids are extremely useful in GVHD; other therapeutics are used only for steroid-resistant GVHD [3,10,11]. Additionally, a number of cancers, such as multiple myeloma and certain lymphomas and leukemias, respond well to combination therapies that include the glucocorticoids prednisone or dexamethasone.

Exposure to high, sustained levels of corticosteroids by any mechanism uncouples the normal metabolic processes from autoregulatory feedback mechanisms and induces a stress response physiological state that cannot be maintained long term without severe consequences. The numerous side-effects experienced by patients administered steroids over the long term are perhaps the clearest example of this. The response to glucocorticoids is complex, due in large measure to the wide variety of physiological contexts in which glucocorticoids act [12]. Complications are time- and dose-dependent and can occur acutely with

very high doses, or more slowly with chronic exposure and lower doses. Fortunately, glucocorticoid-regulated clinical markers of specific side-effects are available from the extensive clinical trials conducted over the years with glucocorticoids. These markers are relevant to many of the impacts of glucocorticoids on bone, fat, and carbohydrate metabolism. Most are readily monitored in response to short-term exposure to glucocorticoids and include serum cortisol, a measure of HPA suppression, lipid profile changes, which measure weight effects, serum glucose and insulin levels, a measure of effects on insulin resistance and hepatic glucose output, and urinary collagen peptides and serum osteocalcin that help assess the impact of compounds on bone metabolism. Together, these markers can assist in the assessment of specific compounds early in clinical trials. The specific side-effects of glucocorticoids can be ranked by patients and physicians. These vary somewhat depending on the person doing the ranking. In particular, patients tend to highlight the physical and mental changes that accompany long-term steroid use. These include fat redistribution and weight gain and steroid-induced psychosis/neurosis. Physicians, on the other hand, tend to concentrate on problems that affect patient medical care, which include hyperglycemia, generalized insulin resistance, as well as suppression of the HPA axis. However, without question, the single most important side-effect from the standpoint of many physicians is osteoporosis. This side-effect alone accounts for an enormous amount of morbidity among patients receiving glucocorticoids. Long-term glucocorticoid treatment often results in some degree of osteoporosis in patients. Because these patients also suffer from decreased muscle mass as a consequence of steroid treatment, they are more susceptible to falling. The consequences of falls and subsequent fractures become enormous when one considers that many of these patients are already sick with a debilitating disease. A hip fracture late in life with its attendant inactivity and increased potential for pneumonia can have fatal consequences for elderly patients. Susceptibility to fractures and aseptic necrosis of the femoral head increases within months of starting glucocorticoid therapy [12,13]. Steroids degrade the quality of trabecular bone, resulting in an increase in fracture rate [14]. Bone loss is highest in the first 6 months of therapy, after which patients continue to lose bone, but at a slower rate. When taken off steroids, patients do appear to partially regain bone [14,15]. The loss of muscle compounds the osteoporosis problem. Glucocorticoid-induced myopathy results in decreased strength and muscle mass. The mechanism by which glucocorticoids affect muscle mass is partially due to hypogonadism observed in many patients with the consequent decline in levels of the sex steroids estrogen and testosterone, which are responsible for contributing to the maintenance of both muscle and bone mass [16,17]. Furthermore, mimicking a stress response, muscle is broken down and utilized as a source of substrates and energy for the increased activity in the glucose production pathways. While beneficial in the short term, this decreases overall muscle function when activated for an extended period of time.

The behavioral effects of glucocorticoids are of great concern to patients. Glucocorticoids have long been known to have psychogenic effects in a subset of patients when given at high doses. Approximately 5% of patients will experience some degree of inappropriate euphoria, psychosis, or depression [18]. Patients are also quite concerned with the effects of glucocorticoids on fat redistribution and weight gain. Fat and muscle are lost from limbs, but truncal and visceral areas actually accumulate fat. Facial, supraclavical, and posterior cervical fat depots are particularly sensitive to glucocorticoids, resulting in the moon face and buffalo hump characteristic of long-term glucocorticoid treatment [19]. Even one dose of a glucocorticoid is sufficient to increase hepatic glucose production and increase insulin resistance of peripheral tissues. The glucocorticoid effect on glycemic control is thought to target insulin signaling [20]. Glucocorticoids affect insulin-mediated increases in blood flow to muscles [21]. They decrease key insulin receptor signaling molecules and increase glucose output by increasing the rate-limiting enzyme in gluconeogenesis, PEPCK [22,23]. Glucocorticoids also inhibit the release of insulin from the pancreas, acting directly on the pancreatic β cells. This action may involve apoptosis of β cell populations, leading to decreased insulin production as well as more direct inhibition of insulin expression [24–26]. The molecular details underpinning regulation of hepatic glucose production have been made clearer recently by discoveries linking GR, other transcription factors, and cyclic AMP (cAMP) in the regulation of the *PEPCK* gene.

3. Molecular biology of the GR

The GR acts as a ligand-regulated transcription factor responding to circulating cortisol. It is a member of the large family of intracellular receptors comprised of both the nuclear hormone receptors as well as the steroid receptor subfamilies. The protein itself is composed of three general domains: a DNA-binding domain, a C-terminal ligand-binding domain (LBD), and an N-terminal activation domain. It is capable of regulating transcription both negatively and positively and is localized to the cytoplasm in the absence of hormone. The receptor is held in an inactive state poised to bind ligand by interaction with a chaperonin complex comprised of heat shock proteins. Upon binding ligand, the receptor undergoes a conformational change that dissociates the heat shock proteins and activates a number of receptor functions including DNA binding activity, nuclear localization, and transcriptional regulation. The latter involves the direct and indirect interaction with a large number of transcription factors critical to gene regulation including RNA polymerase as well

as various polymerase-associated proteins. The complex that forms at a regulated gene is quite large, and the receptor likely does not contact RNA polymerase directly, but instead utilizes several types of so-called ''coactivator'' proteins to bridge the gap between itself and the polymerase [27]. The details of the interaction between the receptor and these coactivators are understood from genetic, biochemical, and crystallographic standpoints for only a few specific receptor—coactivator pairs. In general, these interactions make use of the LBD of the receptor, although other less well-characterized interactions clearly occur in the N-terminal and DNA binding domains [28]. Coactivators bind to the LBD of the GR in a hormone-dependent fashion, interacting directly with the extreme C-terminal portion of the LBD. This interaction domain is formed by the juxtaposition of several helical segments within the protein. The interaction surface between these transcription factors is comprised of a hydrophobic pocket on the receptor and a helical sequence containing an LxxLL amino acid motif on the coactivator [27]. This interaction is very sensitive to the structure of the ligand bound in the pocket. Coactivators typically bind avidly in the presence of agonists, but fail to bind in the presence of antagonist ligands. In fact, this is likely the mechanism of antagonist action. This conformational sensitivity is due to the changes in receptor structure brought about by the structure of the ligand bound in the pocket. The receptor appears to actually condense around the ligand during the binding reaction, meaning that structural changes in the ligand are transmitted directly to the receptor and to the receptor surfaces that interact with coactivators [27]. Thus, ligands can change the ability of the receptor to bind to coactivators. These coactivators may also play a role in the tissue-specific activity of glucocorticoids. Although many coactivators are expressed widely, some have been described that exhibit a restricted tissue expression pattern [29,30]. Coactivators are not only involved in transcriptional activation, but they also appear to play a role in transcriptional repression. Recent data have demonstrated that transcriptional repression by many members of the nuclear hormone receptor family is dependent upon specific corepressor proteins that bind directly to the receptor in the absence of hormone and inhibit the transcription process by recruiting histone deacetylases [31], which appear to condense chromatin and therefore repress transcription [32]. However, most members of the steroid receptor subfamily are sequestered in the cytoplasm in the absence of hormone and exhibit gene-specific repression or activation only when hormone is present. Corepressors have not yet been shown to bind steroid receptors during normal gene regulation [33], although there is evidence for interaction when bound to certain synthetic antagonists [34].

The ubiquitous coactivator GRIP-1 has been shown to bind to GR and other intracellular receptors and to enhance their transcriptional activation activity [35]. Unresolved questions regarding the mechanism of steroid receptor repression prompted a recent analysis of the collagenase-3 gene promoter under glucocorticoid-repressed conditions [36]. Given the previously described role for GRIP-1 as a coactivator, it came as a surprise when these authors demonstrated an important role for GRIP-1 in repression. They demonstrated that GRIP was recruited to the collagenase promoter during the act of repression by the GR. The ligand-dependent recruitment of these proteins might be used as a readout to detect potentially beneficial therapeutic agents.

The search for a novel glucocorticoid that has the anti-inflammatory properties of conventional steroids without one or more of the side-effects has been a long-standing goal of the field. Much effort has been spent on modifying the steroid backbone to achieve this sort of increased therapeutic index; however, these efforts have met with little success. Deflazacort, a D-ring-substituted steroid otherwise similar to cortisol, was touted originally as a powerful anti-inflammatory molecule exhibiting more selective, i.e. reduced, activity, in particular on bone and on glucose metabolism. Initially, clinical data supported this notion [37,38]. However, subsequent trials that adjusted the steroid dose to maintain equivalent anti-inflammatory efficacy usually needed higher levels of deflazacort. Unfortunately, at these higher doses, the advantages of deflazacort disappeared [37]. The field was re-energized by the discovery of the likely mechanism of GR-mediated repression of a wide variety of pro-inflammatory genes. The receptor was shown to bind directly to specific transcription factors (NFκB and AP-1) involved in up-regulating inflammatory genes. This represented a unique mechanism that was genetically separable from transcriptional activation. The search began for ligands that could induce transcriptional repression, but hinder transcriptional activation. In 1997, the first compounds that separated transactivation from transrepression were reported [39].

These compounds were steroidal in nature, were very efficient inhibitors of both AP-1- and NFκB-mediated gene induction, and were strong anti-inflammatory agents *in vivo*. They also were reported to have reduced ability to activate gene expression in some, but not all cellular contexts. Unfortunately, thus far, no *in vivo* therapeutic advantage has been demonstrated for these types of molecules when side-effects were measured [40,41]. This result calls into question the usefulness of the activation–repression hypothesis [40,41].

We have also pursued this hypothesis as an approach to discovering selective GR modulators. An example of the compounds identified during this collaborative effort between Ligand Pharmaceuticals and Abbott Laboratories is AL-438 (Abbott-Ligand 438), which was shown to be a specific, non-steroidal ligand for the GR that exhibited a unique profile, both *in vitro* and *in vivo*. The molecule is fully efficacious at transcriptional repression compared with prednisolone on certain genes related to the anti-inflammatory aspects of glucocorticoid activity (E-selectin

and interleukin-6), but is weaker (a partial agonist) for transcriptional activation. Using other promoters in different cell backgrounds, AL-438 is more active as a transcriptional activator. Thus, AL-438 does not completely separate transcriptional repression from activation, but instead appears to be separating activities in a gene-specific fashion. In animal models, AL-438 was as efficacious as prednisolone at inhibiting inflammation. Importantly, AL-438 exhibited significantly reduced impact on fasting glucose levels compared with prednisolone, suggesting that this compound might not cause the diabetogenic effects of steroidal glucocorticoids.

An as-yet-unanswered question is whether the improved profile of AL-438 *in vivo* is a direct result of its altered effect on GR structure and function as detected by in vitro assays. It is possible that it is the specific conformation of the receptor detected by our in vitro assays that is responsible for the therapeutically beneficial profile observed in rodents.

This is consistent with the gene-specific activity described earlier. The mechanism may be found in the fact that AL-438 generates a receptor conformation that differs from steroids, which in turn changes the spectrum of coactivators with which the receptor can interact. Certain coactivators bind GR with identical affinity in the presence of either AL-438 or prednisolone, while others exhibit significantly reduced affinity in the presence of this compound. We believe that since different genes have different requirements for specific coactivators, this may be the molecular rationale for AL-438's gene-selective profile.

The coactivator GRIP-1 is an example of a potential mediator of gene-specific effects, given its demonstrated involvement in both transcriptional repression and activation. We have examined the interactions between GRIP-1 and GR in a variety of mammalian two-hybrid and GST pull-down assays. Our findings indicate that, in a manner similar to prednisolone, AL-438 will induce the interaction

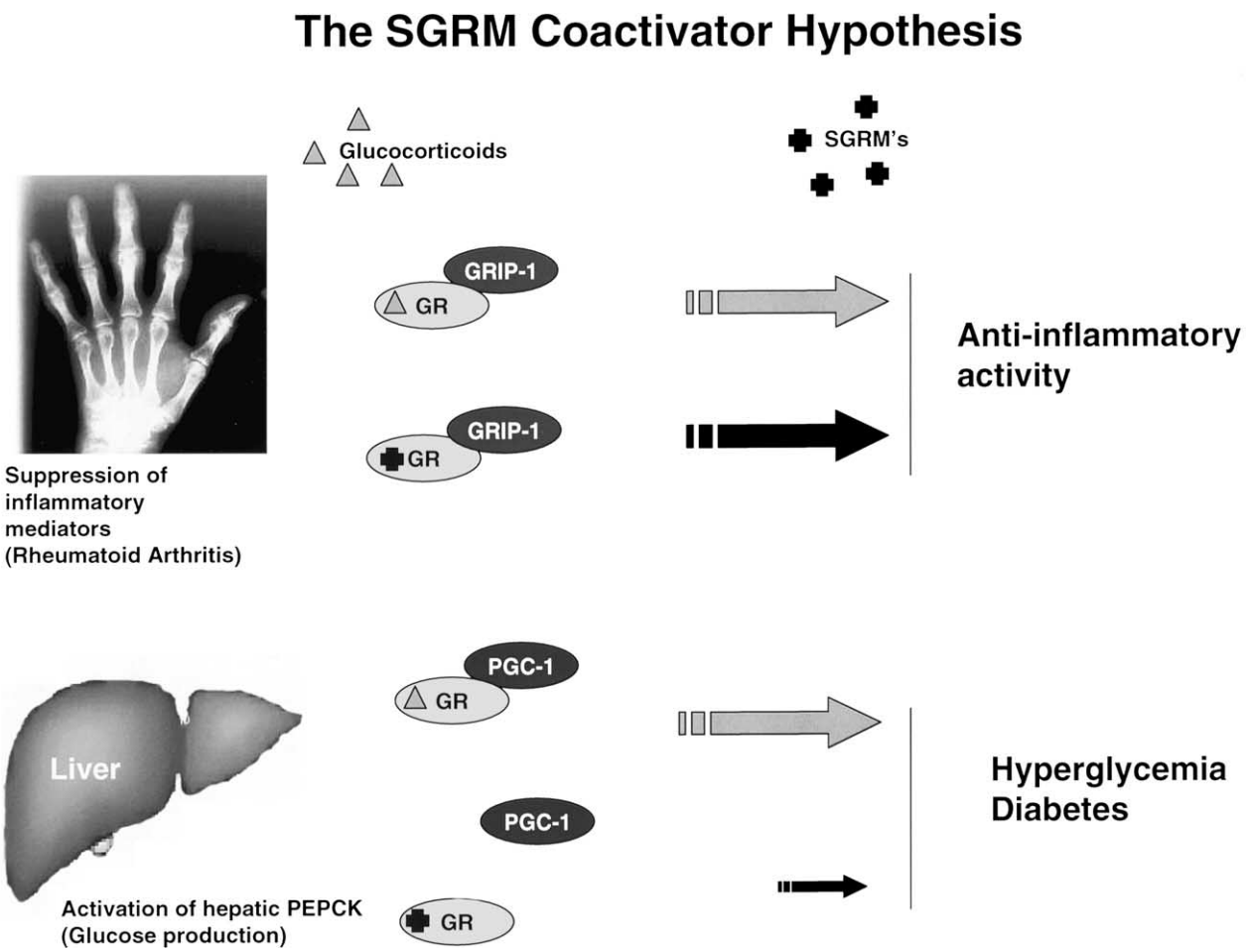

Fig. 1. Selective glucocorticoid receptor modulator (SGRM) coactivator hypothesis. This figure depicts a model that attempts to distinguish the effects of steroidal glucocorticoids from SGRMs. Both compounds are fully efficacious at binding GR and causing the interaction with GRIP-1, a coactivator involved in repression of inflammatory genes by GR. The model suggests that this interaction is, in part, responsible for the anti-inflammatory activity of both compounds. Of course this is likely not the entire explanation. That said, when examining the ability of steroids and SGRMs to induce the interaction with PGC-1, a coactivator involved in glucose homeostasis in the liver among other things, a strikingly different result is found. The SGRM is weaker at inducing the PGC-1 interaction than the steroid. This may help explain the reduced negative impact on glucose metabolism from the SGRM. Thus, the reduced PGC-1 interaction may translate into reduced side-effects in liver and possibly other tissues as well.

between GRIP and GR efficiently. This finding suggests that the differences between AL-438 and steroids *in vivo* are not to be explained by the GRIP-1—GR interaction, with the caveat that we have not tested all cell contexts with this approach. Another example of potential coactivators for the GR is the PGC-1, originally characterized as a PPAR coactivator highly expressed in brown fat, involved in fat differentiation [30], and more recently shown to play a critical role in glucocorticoid-mediated stimulation of glucose production from the liver. The *PEPCK* gene requires the action of PGC-1 together with the transcription factor HNF-4 to efficiently respond to glucocorticoids [29,42,43]. These authors demonstrate that PGC-1 binds directly to GR and to HNF-4 and is critical for the appropriate response to both cAMP signaling and glucocorticoids. We became interested in this protein initially because of the critical role played by PGC-1 in fat metabolism and differentiation [30], an activity intimately tied to the effects of glucocorticoids. The PGC-1—GR connection was strengthened considerably by the demonstration of direct binding to GR [44] and most recently by its involvement in glucocorticoid effects on glucose production [42,43]. Thus, we hypothesized that the reduced impact of AL-438 on fat metabolism and glucose levels compared with prednisolone might be connected to changes in the interaction with PGC-1. This notion was tested with direct interaction assays that demonstrated that PGC-1 could bind directly to the GR in response to prednisolone and dexamethasone. The response to AL-438 in the same assays was reduced, suggesting that, in contrast to GRIP-1, AL-438 did not induce a GR conformation that efficiently interacted with PGC-1. Fig. 1 outlines the hypothesis that these data supported.

This idea suggested that for the inhibition of inflammatory mediators (collagenase, interleukin-6, and E-selectin), interaction with GRIP-1 was important [36], and both AL-438 and steroids induce this interaction efficiently. In contrast, the interaction with PGC-1 is known to be important for the effects of glucocorticoids on liver glucose production and hyperglycemia [42,43]. AL-438 is less efficient at inducing PGC-1 interactions when compared with steroids, perhaps explaining the differences between the compound and the steroids on glucose levels *in vivo*. This hypothesis remains to be tested by directly demonstrating the involvement of PGC-1 and GRIP-1 in the differential activity of AL-438. Furthermore, the differential interactions induced by these compounds may extend to other coactivators.

For the past 30 years, efforts to develop new, more powerful, and safer anti-inflammatory agents that work through the GR have concentrated mainly on the steroid backbone. Recent discoveries of the molecular and cellular aspects of GR activity have opened several exciting new approaches to this effort. Indeed, recent advances in chemistry and screening, together with new molecular approaches, have allowed the identification of novel non-steroidal compounds that exhibit activities that have the promise of leading to powerful, yet safer GR-dependent anti-inflammatory agents.

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