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Ligand structural motifs can decouple glucocorticoid receptor transcriptional activation from target promoter occupancy

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Abstract

Glucocorticoid (GC) induction of the tyrosine aminotransferase (TAT) gene by the glucocorticoid receptor (GR) is a classic model used to investigate steroid-regulated gene expression. Classic studies analyzing GC-induction of the TAT gene demonstrated that despite having very high affinity for GR, some steroids cannot induce maximal TAT enzyme activity, but the molecular basis for this phenomenon is unknown. Here, we used RT-PCR and chromatin immunoprecipitation to determine TAT mRNA accumulation and GR recruitment to the TAT promoter (TAT-GRE) in rat hepatoma cells induced by seven GR ligands: dexamethasone (DEX), cortisol (CRT), corticosterone (CCS), 11-deoxycorticosterone (DOC), aldosterone (ALD), progesterone (PRG) and 17-hydroxyprogesterone (17P). As expected, DEX, CRT, CCS and ALD all induced both TAT mRNA and GR recruitment to the TAT-GRE, while PRG and 17P did not. However, while DOC could not induce significant TAT mRNA, it did induce robust GR-occupancy of the TAT-GRE. DOC also induced recruitment of the histone acetyltransferase p300 to the TAT-GRE as efficiently as DEX. These DOC-induced effects recapitulated at another GR target gene (sulfonyltransferase 1A1), and DOC also failed to promote the multiple changes in gene expression required for glucocorticoid-dependent 3T3-L1 adipocyte differentiation. Structural simulations and protease sensitivity assays suggest that DOC and DEX induce different conformations in GR. Thus, although steroids that bind GR with high affinity can induce GR and p300 occupancy of target promoters, they may not induce a conformation of GR capable of activating transcription.

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Keywords

glucocorticoid receptor; anti-inflammatory; steroids; chromatin immunoprecipitation; gene expression

Introduction

Glucocorticoids (GCs) are steroid hormones that regulate development, metabolism and the stress response [1]. GCs are also used therapeutically as potent anti-inflammatory agents for the treatment of arthritis, asthma and leukemia [2]. Problems arise when GCs are administered chronically, which can result in bone and muscle atrophy, stunted growth, and increased susceptibility to diabetes [3]. Developing GCs and non-steroidal analogs that minimize these harmful side effects while maintaining anti-inflammatory activity remains an important goal of pharmaceutical research [4].

Glucocorticoids elicit their physiological responses via the glucocorticoid receptor (GR), a ligand activated transcription factor [5]. Upon ligand binding, GR translocates into the cell nucleus where it can bind glucocorticoid-response elements (GREs) in the regulatory regions of target genes, enhancing transcription by interacting with coactivators that attract chromatin remodeling factors, such as p300, which in turn facilitate the recruitment of the RNA polymerase II transcription initiation complex [6; 7]. GR also regulates gene expression independent of DNA-binding through protein-protein interactions, exemplified by GR repression of NF κ B and AP-1 stimulated genes, two pathways associated with the anti-inflammatory activities of GCs [8; 9].

Tyrosine aminotransferase (TAT) is an enzyme expressed in the parenchymal cells of liver where it is involved with the transamination of xenobiotics to aid in excretion and in the metabolism of several classes of drugs [10; 11]. GCs induce the TAT gene, which is controlled by a regulatory region located ~2.5 kb upstream of the start site of transcription [12; 13]. This region consists of two clusters of GRE's referred to as GC-responsive units I and II (GRU I, ²GRU II). Mutagenesis studies *in vitro* and GR recruitment *in vivo* have demonstrated that GC-mediated TAT induction occurs mainly through the GRU I regulatory region [13; 14].

TAT was one of the first gene products ever shown to be stimulated by GCs in cultured cells [15] and has become a model for GC-regulated transcriptional regulation. Classic studies by Samuels and Tomkins established a hierarchy of the efficacy and potency of the induction of TAT enzyme activity by various steroids [16]. These studies and others showed that there was no clear correlation between the affinity of a particular steroid for GR and its ability to activate maximal TAT enzyme activity [16; 17]. For example, despite corticosterone and 11-deoxycorticosterone having similar affinities for GR ($K_d = 7.0$ and 9.1 nM, respectively), deoxycorticosterone is only capable of inducing 50% of TAT activity relative to DEX, whereas corticosterone can induce 100% DEX TAT activity [16]. These results suggested that mechanisms apart from ligand binding to receptor were modulating TAT induction by different steroids. To gain further insight into the general mechanism of gene induction by steroids, we determined the mRNA induction, GR and p300 recruitment induced by seven representative steroids.

Materials and Methods

Cell culture

GRH2 rat hepatoma cells stably expressing rat GR (clone G10) [18] were maintained in Dulbecco's modification of Eagle's media (DMEM) with 2 mM L-Glutamine (Mediatech/Cellgro, Herndon, VA) supplemented with 10% Fetal Bovine Serum (FBS; HyClone, Logan, UT), and 50 units/ml Penicillin and Streptomycin (Mediatech/Cellgro). 3T3-L1 pre-adipocytes (ATCC) were cultured in DMEM with 10% FBS at low density. Confluent cultures were differentiated by the addition of 1 ug/mL insulin, 0.5 mM isobutyl-1-methylxanthine, and 10nM or 100nM dexamethasone, or deoxycorticosterone and cells were maintained in this medium until processing for analysis.

Ligand Treatments

Rat hepatoma cells in 15 cm plates containing 25 ml DMEM with 10% FBS were cultured until they reached ~ 95% confluence and were then treated with indicated ligand concentrations. For ligand treatments that represent the EC₅₀ for induction of TAT enzyme activity [16]: Dexamethasone 8nM; Cortisol 50nM; Corticosterone 25nM; Deoxycorticosterone 600nM; Aldosterone 400nM; Progesterone 4000nM; 17-Hydroxyprogesterone 4000nM.

Reverse Transcriptase PCR

The *C. therm.* one-step RT-PCR kit (Roche Diagnostics, Inc.) was used for all RT-PCR reactions. Total RNA from hepatoma cells was isolated using the RNeasy kit (Qiagen), and eluted from RNA purification columns. RNA concentration was determined by optical density and diluted to 0.25 ng/μl. RT-PCR reactions for *tat*, *sult1a1* and *gapdh* were performed on 0.05 ng total RNA. The PCR products were separated on a 3% agarose gel, visualized by ethidium bromide staining and photographed under UV light. Images of the *tat* and *sult1a1* expression products were scanned and quantitated using NIH Image (<http://rsb.info.nih.gov/nih-image/>), and normalized to *gapdh* signals.

Chromatin Immunoprecipitation (ChIP) Assays

Performed precisely as previously described [19], sequences of each primer pair are: *tat* GRE forward 5' - CCC ATA AAT AAC AGG AAG CCC AAG -3'; *tat* GRE reverse 5' - ATA CCA CCA CAC CCA GAA ACC GAC -3'; *tat* DOWNSTREAM forward 5' -TGT TCT AGC AGG ATT TGT CTG TG -3'; *tat* DOWNSTREAM reverse 5' -TCT GAG CCT GTT GTT GGC CCA -3'; *sult1a1* GRE forward 5' - CGT GTG AAT GCT CTG TCC CAT C -3'; *sult1a1* GRE reverse 5' - GCT GAA GAT TTT GTG TCC CAA GTG -3'; *sult1a1* UPSTREAM forward 5' - GTC TTC TCC TAA TCC ACA AAT CCG -3'; *sult1a1* UPSTREAM reverse 5' - GGG GTG CTG AAA ACA AAT AAC CAC -3'

Oil Red O staining

3T3-L1 cells were washed three times with PBS and fixed in 10% formalin in PBS for 1 h. After washing the cells twice with PBS, cells were stained with Oil Red staining solution (0.5% Oil Red [Sigma] in isopropanol, diluted 3:2 in water and filtered with a 0.22-um filter). After staining, cells were washed three times with water and photographed.

Molecular modeling of GR:DOC

The initial structure [pdb code: 1M2Z.pdb [20] of human GR:Dexamethasone (DEX) and human MR:deoxycorticosterone (DOC) [pdb code: 2AA7.pdb [21] complexes were retrieved from the Protein Databank (PDB, <http://www.rcsb.org/pdb>). The ICM program was used for protein and ligand docking (Molsoft LLC, San Diego, CA). The coordinates of

the GR structure were converted into an ICM project. The molecular structure of DOC was extracted from the MR:DOC complex. Atom and bond types of DOC were corrected in ICM interface. The geometry of DOC was optimized with MMFF94 force field [22]. The DOC molecule was docked into the ligand-binding pocket with default parameter implemented in ICM

Protease sensitivity assays

Rat GR was synthesized *in vitro* using with TnT T7 quick-coupled transcription/translation system (Promega Corp. Madison, WI) in the presence of ^{35}S -methionine as described by the manufacture. Translation mix (10 μL) was diluted into 40 μl of 50 mM Tris-HCl pH 7.5 containing 100 nM Dex, 100 nM DOC or ethanol. The samples incubated overnight at 4°C. Serial 10-fold dilutions of 1 mg/ml porcine pancreatic elastase (Sigma, E7885) were added to 4 μl of the sample diluted into 36 μl of 50 mM Tris-HCl pH 7.5 and incubated for 4 min at room temperature and terminated by the addition of 7.5 μl of 5X SDS sample buffer, boiled and resolved by 10% SDS-PAGE. Gels were fixed (25% Methanol, 10% acetic acid), dried and radiolabel polypeptide fragments visualized by autoradiography.

Results

We selected seven representative steroids to characterize, based on previously determined pharmacological properties [16; 17]. Figure S1 shows the structures of these seven compounds and lists the previously determined values for their dissociation constants for GR binding (K_d), the EC_{50} s for TAT enzyme induction (EC_{50}), and their intrinsic activity to activate TAT enzyme relative to Dexamethasone (TAT IA), the standard synthetic GR agonist. We noted that there is or TAT IA for these compounds, which led us to no clear correlation between GR binding, EC_{50} further characterize how these steroids induce the TAT gene.

Using a rat hepatoma cell line, we examined induction of TAT mRNA by these steroids. of TAT induction determined previously for each steroid [16]: Cells were treated 3hrs at the EC_{50} 17P (4 μM), PRG (4 μM), DOC (600nM), CCS (25nM), CRT (50nM), ALD (400nM) or DEX (8nM). Total RNA was isolated and subjected to RT-PCR and the products separated by agarose gel electrophoresis. As expected ETH, 17P, PRG and DOC did not induce significant accumulation of TAT mRNA, while CCS, CRT, ALD and DEX all increased TAT mRNA levels (Figure 1A). These results are largely consistent with the induction of TAT enzymatic activity by these steroids [16], however the lack of significant DOC-induced TAT mRNA lead us to repeat these experiments using saturating concentrations for all the compounds, which yielded similar results (data not shown). To determine whether GR is specifically recruited to the TAT promoter upon steroid treatment, ChIP assays were performed (Figure 1B) after treating rat hepatoma cells with steroids identically as in Figure 1A. Consistent with the TAT mRNA expression data, we observed that ETH, 17P and PRG failed to induce significant recruitment of GR, while CCS, CRT, ALD and DEX all induced GR recruitment to the TAT-GRE (Figure 2B). However, DOC induced robust recruitment of GR to the TAT-GRE, equal to CCS and DEX, yet TAT mRNA induction by DOC was almost undetectable (refer to Figure 1A). We observed similar results using saturating concentrations for all compounds (data not shown). The lack of DOC-induced TAT mRNA accumulation was not due simply to variations in the kinetics of TAT induction by DOC vs. DEX, since we did not detect significant levels of TAT mRNA accumulation upon DOC treatment throughout a 72-hour treatment regimen (Figure 1C).

We next determined if there were any changes in the recruitment of the transcriptional cofactor p300 induced by DOC and DEX to the TAT-GRE. p300 is a cofactor central to

transcription initiation that functions in histone acetylation [23]. We treated rat hepatoma cells with DOC or DEX, and examined p300 occupancy by ChIP. Surprisingly, recruitment of p300 to the TAT-GRE was very similar between DOC and DEX treatments at 1hr and 3hrs of treatment (Figure 1D). Taken together, these data demonstrate that although DOC induces GR to occupy the TAT-GRE, GR is not competent to induce TAT mRNA, despite the presence of p300 at the TAT promoter.

We next asked if the effect of DOC on p300 recruitment and RNA induction were specific to the TAT gene, or if this phenomenon is observable at another GC-inducible GR target gene, Sulfonylesterase 1A1 (*sulta1a1*) [19]. Indeed, DOC fails to induce *sulta1a1* mRNA over a range of concentrations (Figure 2A), yet p300 is recruited to the GRE of the *sulta1a1* promoter in response to DOC as efficiently as DEX (Figure 2B). These data demonstrate that our observations at the TAT promoter are not particular only to the TAT gene, at least in rat hepatoma cells.

We then asked if DOC could drive GR-dependent adipocyte differentiation, which is a more complex physiological readout reflecting multiple changes in gene expression driven by GR. Using mouse 3T3-L1 pre-adipocytes, where DEX, insulin, and 3-isobutyl-1-methylxanthine (IBMX) act together to drive adipocyte differentiation, we assessed the ability of DOC to replace DEX in the differentiation cocktail. DEX induced adipocyte differentiation, as assayed by Oil Red O staining (a measure of intracellular fat accumulation), whereas DOC failed to induce differentiation (Figure 2C). Other steroids that induce TAT transcription were also able to promote differentiation similar to DEX (data not shown). These data demonstrate that DOC is unable to induce GR-dependent adipocyte differentiation in 3T3-L1 cells.

Phosphorylation of GR has been shown to modulate GR transcriptional activity [24]. However, we did not observe any significant differences in DOC vs. DEX-induced GR phosphorylation at serines 203, 211 and 226 (Figure 3A), nor are there any differences in the chromatin recruitment of specific GR phospho-isoforms to the TAT-GRE by ChIP (Figure 3B). These data suggest that DOC and DEX do not induce different phosphorylations on GR, and so phosphorylation of GR is not likely the mechanism accounting for differences in target gene induction.

Molecular docking was then performed to determine how DOC binds to GR. Comparison of the GR:DEX structure (Figure 4A) and our GR:DOC model (Figure 4B) reveals that both ligands make similar interactions with GR, and DOC fits well within the GR ligand-binding pocket. The most significant difference lies in the hydroxyl group at the C-11 position of the steroid C-ring, which is absent in DOC relative to DEX. In the GR:DEX structure, the amide side chain of N564 in helix 3 simultaneously forms hydrogen bonds with C-11 hydroxyl group of DEX and backbone carbonyl of E748, which is present in a loop preceding the C-terminal activation function 2 (AF-2) helix. This hydrogen bond network induced by DEX stabilizes helix 3 and positions the AF-2 helix in an “active” conformation. The GR:DOC model predicts that this hydrogen bond network will not form, altering the induced structure of GR. Based on this model, we hypothesize that DOC induces a GR conformation that is distinct from the DEX-bound form, which correlates with reduced GR transcriptional activation.

We next probed protease sensitivity differences between GR:DEX vs. GR:DOC. Radiolabeled GR was synthesized *in vitro*, bound with 100nM DEX, DOC or left unbound (EtOH), then digested with increasing amounts of elastase. DEX produced a distinct digestion pattern of GR relative to the unbound control (Fig 4B; compare lanes 2–5 to 10–13), with a prominent protected fragment at the highest elastase concentration. When bound

to DOC, GR showed an increase in protection from proteolytic degradation compare to control, but less than that observed with DEX (compare lanes 3–5 and 7–9). Additionally, the stable fragment in the GR:DEX complex at the highest elastase concentration is not observed in DOC bound GR (arrowhead, compare lanes 5 and 9). Thus, these data demonstrate that DOC-bound GR induces an elastase digestion pattern between that of DEX and the no-hormone receptor, suggesting that DOC and DEX induce distinct GR conformations.

Discussion

The regulation of TAT activity by steroids has been extensively studied, and is a paradigm for steroid-induced gene expression. Our findings indicate that TAT mRNA induction and GR recruitment to the TAT-GRE largely parallels the induction of TAT enzyme activity by the steroids examined. Surprisingly, DOC induces GR and p300 recruitment to the TAT-GRE as efficiently as DEX, but fails to induce significant TAT mRNA expression. Homology modeling and protease digestion experiments suggest that DOC and DEX induce different conformations in GR. Thus, DOC binding changes the transcriptional regulatory activity of GR at a step downstream of receptor and p300 recruitment, perhaps through different GR conformations induced by DOC *vs.* DEX.

How DOC induces GR and p300 recruitment to TAT-GRE to the same extent as DEX, yet does not cause mRNA induction remains an unanswered question. DOC differs from DEX at 5 chemical positions on the steroid ring, including the lack of a hydroxyl at the C-11 position of the steroid C-ring in DOC (Figure S1). In the GR:DEX crystal structure, this particular hydroxyl group makes a critical contact with N564 of GR [20]. Mutation of N564 to alanine in GR results in a substantial decrease in DEX-induced receptor transcriptional activity, suggesting that the C-11 hydroxyl interaction with N564 contributes to stabilization of the activated conformation of GR [25]. These observations of GR:DEX are consistent with our structural simulations of GR:DOC (Figure 4A), however the other chemical differences between DOC and DEX could also contribute to alter GR activity.

GRIP1 is a known GR coactivator, but it has also been shown that GRIP1 can function as a GR corepressor at certain GREs [26] though a domain distinct from the GRIP1 activation domain [27; 28]. It is tempting to speculate that the conformation of GR:DOC at the TAT-GRE may facilitate the GRIP1 corepressor conformation. This might help explain why GR:DOC has a dominant-inhibitory effect on TAT gene expression, despite the presence of GR and p300 at the TAT-GRE.

There have been several reports of nuclear receptor ligands being capable of inducing receptor recruitment while being transcriptionally silent [29; 30; 31; 32]. Dissociation of GR recruitment and activation has also been observed for arylpyrazole compounds, which can induce GR occupancy at target genes, but were inactive in transcription [33]. It is interesting to note that a synthetic GR ligand (AL-438) that retains anti-inflammatory efficacy without affecting bone and glucose metabolism, reduces the interaction between GR and peroxisomal proliferator-activated receptor coactivator-1 (PGC-1), while maintaining GRIP1 interaction [34].

Our data also suggest that lead anti-inflammatory compounds could be designed to occupy the promoter of GR target genes without activating transcription. This activity could enhance anti-inflammatory efficacy by competing with “transcriptionally active” GR for these promoters. It is very important to note that progesterone, despite having reasonable affinity for GR ($K_d = 50\text{nM}$) and only differing from DOC by a single atom (Figure S1), cannot induce GR recruitment to TAT-GRE (Figure 1B). It therefore seems possible to

define structure-activity relationships (SARs) that result in high-affinity binding to GR, yet a receptor that is transcriptionally silent. Given that many of the deleterious side effects of GCs manifest through DNA-binding dependent GR activation of target genes containing classic GREs in their promoters [4], this type of SARs analysis could help focus anti-inflammatory lead development into active, but transcriptionally silent compounds. This could aid in minimizing the side effects of anti-inflammatory compounds currently under development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GC	glucocorticoid
GR	glucocorticoid receptor
TAT	tyrosine aminotransferase
DEX	dexamethasone
CRT	cortisol
CCS	corticosterone
DOC	11-deoxycorticosterone
ALD	aldosterone
PRG	progesterone
17P	17-hydroxyprogesterone
GRE	glucocorticoid response element
GRU	glucocorticoid responsive unit

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Highlights

11-deoxycorticosterone (DOC) induces GR & p300 occupancy of two target genes.

DOC does not induce mRNA of these two genes

DOC fails to induce complex glucocorticoid-dependent adipocyte differentiation.

Protease sensitivity & modeling show DOC & DEX change GR structure.

Figure 1. DOC induces GR recruitment to TAT without inducing mRNA

A of TAT, Rat hepatoma cells were treated with ethanol or indicated steroid for 3h at the EC_{50} activation [16], total RNA isolated and subjected to RT-PCR with primers specific for the TAT and GAPDH mRNAs, quantitation on right. **B**, Cells treated with steroids identically as in A, then subjected to ChIP using a polyclonal anti-GR antibody. Precipitates were amplified using primers flanking the GRE necessary for TAT activation by GCs (TAT-GRE), or to a region ~ 5 kb downstream of the TAT promoter (control), quantitation on right. **C**, Rat hepatoma cells were treated with 600nM DOC or 8nM DEX for 0,1,12,24,48 or 72 h. Total RNA isolated and subjected to RT-PCR as in A. **D**, Rat hepatoma cells treated with 600nM DOC or 8nM DEX over indicated time course subjected to ChIP using polyclonal anti-p300 antibodies, precipitates then amplified using primers flanking TAT-GRE. Input represents 0.5% of the total chromatin. All experiments repeated three times with similar results, error bars in this and all figures represent standard error of the mean.

Figure 2. DOC effects on GR are not specific to TAT

A, Rat hepatoma cells treated with indicated concentrations of DOC or DEX for 24 hours, total RNA isolated and subjected to RT-PCR with primers for *sult1a1* and *gapdh* mRNA, results quantitated relative to *gapdh*, repeated twice with similar results. **B**, Rat hepatoma cells were treated with 10nM DEX or DOC over 3h, fixed with formaldehyde and subjected to ChIP using polyclonal anti-p300 antibodies. Precipitates were amplified using primers flanking the GRE in the *sult1a1* promoter. Input represents 0.5% of the total chromatin, experiment repeated three times with similar results. **C**, Confluent cultures of 3T3-L1 cells were placed in differentiation medium that contained IBMX, insulin with DMSO (CTRL), DEX, and DOC at indicated concentrations and stained with Oil Red.

Figure 3. DOC and DEX induce similar phosphorylation and TAT recruitment of GR-phospho-isoforms

A, Western blots of rat hepatoma whole cell extracts after 3 hours of treatment with EtOH, 600nM DOC or 8nM DEX, probed as indicated on the left. **B**, Rat hepatoma cells treated as in A, subjected to ChIP using antibodies indicated on the left.

Figure 4. Changes in GR conformation induced by DOC and DEX

A, DEX (X-ray structure, 1M2Z.pdb) and **B**, DOC (docked molecular simulation) are shown with yellow-color carbon atoms. Dashed lines show critical hydrogen bond networks. **C**, ³⁵S-methionine-labeled rat GR was synthesized in an *in vitro* transcription/translation system in the presence of ethanol or 100nM DEX or DOC and incubated with increasing amounts of elastase. The polypeptide fragments were separated by SDS-PAGE, fixed, dried and exposed to x-ray film.