Real-time analysis of gene regulation by glucocorticoid hormones

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Abstract

There is increasing evidence that temporal factors are important in allowing cells to gain additional information from external factors, such as hormones and cytokines. We sought to discover how cell responses to glucocorticoids develop over time, and how the response kinetics vary according to ligand structure and concentration, and hence we have developed a continuous gene transcription measurement system, based on an interleukin-6 (IL-6) luciferase reporter gene. We measured the time to maximal response, maximal response and integrated response, and have compared these results with a conventional, end point glucocorticoid bioassay. We studied natural glucocorticoids (corticosterone and cortisol), synthetic glucocorticoids (dexamethasone) and glucocorticoid precursors with weak, or absent bioactivity. We found a close correlation between half maximal effective concentration (EC50) for maximal response, and for integrated response, but with consistently higher EC50 for the latter. There was no relation between the concentration of ligand and the time to maximal response. A comparison between conventional end point assays and real-time measurement showed similar effects for dexamethasone and hydrocortisone, with a less effective inhibition of IL-6 seen with corticosterone. We profiled the activity of precursor steroids, and found pregnenolone, progesterone, 21-hydroxyprogesterone and 17-hydroxyprogesterone all to be ineffective in the real-time assay, but in contrast, progesterone and 21-hydroxyprogesterone showed an IL-6 inhibitory activity in the end point assay. Taken together, our data show how ligand concentration can alter the amplitude of glucocorticoid response, and also that a comparison between real-time and end point assays reveals an unexpected diversity of the function of glucocorticoid precursor steroids, with implications for human disorders associated with their overproduction.

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Introduction

Glucocorticoid hormones exert a wide diversity of effects in target tissues. Their activity has been typically explored using a limited number of timed end points, both in vivo and in vitro, and using such approaches a variety of synthetic analogues have been developed for use in inflammatory conditions (Hillier 2007). Some agents, such as dexamethasone, show increased selectivity of action towards glucocorticoid pathways as opposed to mineralocorticoid (Hillier 2007).

It has recently become clear that even minor changes to the structure of a ligand can result in a distinct and unpredictable pattern of activity (Wang et al. 2006). However, the effects of a steroid can also be altered by varying its effective biological half-life, as for dexamethasone (Samtani & Jusko 2005, 2007). In vivo, the pharmacokinetics of steroids has been exploited to introduce topical activity, by, for example, making steroid structures susceptible to metabolism (e.g. budesonide; Ryrfaåldt et al. 1982, Brunner et al. 2006, Singh et al. 2007).

It is now possible to track target gene promoter activity continuously, in real-time, without affecting cell viability. This approach allows, for the first time, temporal deconvolution of the effects of steroid, permitting robust measurement of the time to onset of effect, maximal effect, integrated effect and resolution phase. The luciferase reporter used in these studies is unstable, and hence luciferase activity is dependent on new gene transcription, a significant advantage over more stable reporter gene products, such as fluorescent proteins (Takasuka et al. 1998).

We have been able to establish a robust and sensitive assay of glucocorticoid action using the repression of interleukin-6 (IL-6) promoter activity. IL-6 is a physiologically relevant endogenous glucocorticoid receptor (GR) target gene and is important in both the innate immune response and the elaboration of the systemic response to inflammation (De Bosscher et al. 2005). We were able to show that three well-characterised glucocorticoid molecules all had the expected ability to repress IL-6 transcription, and that three closely related steroids did not. Furthermore, we were able to distinguish between the concentration–maximal response, the concentration–integrated response and the concentration–independent time to maximal response for each steroid. This approach allows, for the first time, temporal deconvolution of the effects of steroid, permitting robust measurement of the time to onset of effect, maximal effect, integrated effect and resolution phase. The luciferase reporter used in these studies is unstable, and hence luciferase activity is dependent on new gene transcription, a significant advantage over more stable reporter gene products, such as fluorescent proteins (Takasuka et al. 1998).

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The expected rank order of steroid potency was not observed using this assay, as hydrocortisone was found to be significantly more active than either corticosterone or dexamethasone. This study emphasises the need to consider each specific effect of the steroid independently.

Materials and Methods

Plasmid construction

The promoter region of human IL-6 was amplified from human genomic DNA using the HiFi PCR kit (Roche) following the manufacturer’s instructions. The primers were designed to amplify from within the transcriptional start site to the upstream promoter region yielding a 3000 bp fragment (IL-6). The primers for 3000 bp IL-6 product are as follows:

FWD primer (IL-6F1): 5′CAGATCCAGCAGCA-CAGGAAG3′
REV primer (IL-6R1): 5′GATAGAGCTTCTCTTTCT-GTTCC3′

Upon successful purification, the DNA was adenylated with the addition of an A overhang to the 3′-end, allowing for the subsequent ligation into the pCR2.1-TOPO subcloning vector (Invitrogen). Inserts ligated in the correct orientation in the TOPO TA vector were then cut out using restriction enzymes EcoRV and KpnI. This would allow the insert to be subsequently ligated in the destination vector, PGL4-basic (Promega), in the correct orientation due to the presence of the same restriction enzyme sites.

The PGL4-basic vector was digested using EcoRV and KpnI. Both the PCR fragments and the newly cut vector were then gel-extracted as before, and then each fragment was ligated into the PGL4-basic vector using T4 ligase (Roche). TOP 10 cells were transformed with the new constructs, and grown as a single colony expansion. The constructs were then isolated and purified using a MiniPrep Kit (Qiagen). The constructs were externally sequenced throughout the promoter fragment region (LARK Technologies, Thakeley, Essex, UK).

Cell culture and stable transfection

Rat-1 cells, a rat fibroblast cell line known to express glucocorticoid receptor, and to be glucocorticoid responsive, were cultured at 37 °C (5% CO2) in high-glucose Dulbecco’s modified Eagle’s medium; (DMEM; catalogue no. 11965-092, Gibco) supplemented with 10% FBS (Gibco), and a mixture of penicillin–streptomycin–glutamine (PSG from Gibco no. 10378-016).

Stable transfection of Rat-1 cells was performed using Fugene6 according to standard protocols. The transfected cells were selected in 200 µg/ml hygromycin for 2 weeks after which resistant clones were frozen at −80 °C until required for further experiments. No phenotypic changes or growth rate alterations were induced by the DNA construct.

End point luciferase assay

Approximately 1×10⁶ cells were seeded in a 35 mm dish 4 days before the experiment and allowed to grow to confluence. The medium was changed and drug treatments given. Treatments included dexamethasone (D1756; Sigma) with a final concentration range from 0.1 to 1000 nM, 5 ng/ml tumour necrosis factor α (TNFα; Calbio, Nottingham, UK), 1000 nM progesterone (Sigma–Aldrich), 1000 nM pregnenolone acetate (Sigma–Aldrich), 1000 nM of 21-hydroxyprogesterone-21-acetate (Sigma–Aldrich), 1000 nM corticosterone (Sigma–Aldrich), 1000 nM hydrocortisone and 1000 nM of 17-hydroxyprogesterone (Sigma–Aldrich).

After 24 h, luciferase assays were performed according to the manufacturer’s instructions (Promega). Briefly, the medium was aspirated from the wells and 200 µl lysis buffer was added. To 100 µl lysate was added 50 µl beetle luciferin substrate (0.1 mM total luciferin) and the bioluminescence was measured using the Mithras LB940 automated analyser (Berthold Technologies East Grinstead, UK).

Real-time luciferase assay

Approximately 1×10⁶ cells were seeded in a 35 mm dish 4 days before the experiment and allowed to grow to confluence. The medium was then replaced with the real-time assay medium (serum-free DMEM without phenol red, catalogue no. 13000-021, Gibco) supplemented with bicarbonate (350 mg/l), 10% FBS and 10 mM HEPES (pH 7.2), antibiotics (25 units/ml penicillin, 25 µg/ml streptomycin) and 0.1 mM luciferin (Promega). The 35 mm Petri dishes are sealed with coverslips and silicon grease. The cells were assayed using a custom-made apparatus based on that developed by Takahashi et al. with luminescence measured using Hamamatsu photon counting modules (Yoo et al. 2004).

After 48 h, the cells were removed from the photomultiplier tube and treated with the compounds directly in the growth medium. The dishes were immediately resealed and replaced into the photomultiplier tubes. Treatments included dexamethasone (D1756; Sigma) with final concentration range from 0.1 to 1000 nM, 5 ng/ml TNFα (Calbio), 1000 nM progesterone, 1000 nM pregnenolone acetate, 1000 nM of 21-hydroxyprogesterone–21-acetate, 1000 nM corticosterone, 1000 nM hydrocortisone and 1000 nM of 17-hydroxyprogesterone.

Photomultiplier tube data analysis

Data were analysed to determine the effects of the various drug treatments. A logarithmic trend line was fitted using the data obtained for 48 h before the commencement of treatment. This procedure removed any baseline non-stationarities from the data.
analysis. This trend line was then subtracted from the log values of the treatment period. The effect of compound treatment was calculated using deviation from the trend line (Fig. 1). The total level of induction/inhibition was calculated as the total sum of deviation from the trend line.

Statistical analysis

Comparisons between groups were by ANOVA followed by Bonferroni’s t-test.

Results

Kinetic deconvolution of glucocorticoid response

In analyzing the effects of glucocorticoids *ex vivo*, a single end point is analyzed at a limited number of time points. However, the integrated glucocorticoid effect is determined by both the amplitude of response and its duration. In order to track the glucocorticoid response in real time, a continuous, non-destructive cell-based assay was developed (Fig. 1). The cells remain viable in this environment for up to 10 days (data not shown), and the complete time course of glucocorticoid response can be followed, allowing an accurate measurement of the time to onset, duration, amplitude and integrated response (area under the curve) to be determined (Fig. 1).

**TNFα effects on IL-6-luc in real-time**

TNFα is a major physiological regulator of the IL-6 gene expression. To determine whether TNFα was also capable of appropriately regulating the IL-6-luc reporter cell, the effects of TNFα alone and those of dexamethasone were measured.
Steroid specificity of IL-6 response

To confirm that the IL-6 response was specific to those steroids with proven agonist activity at the glucocorticoid receptor, a panel of steroids, including glucocorticoid precursors, was analysed. As expected, the three known agonists, hydrocortisone, corticosterone and dexamethasone, were all active in the assay, but hydrocortisone showed a significantly greater overall integrated inhibition than either dexamethasone or corticosterone (Fig. 4a). In contrast, 21-hydroxyprogesterone had a weak effect that failed to reach significance, and pregnenolone appeared to stimulate IL-6 promoter activity, as determined by an inverse excursion of the measurements from the logarithmic trend line (Fig. 4a). The time delay to maximal inhibition did not differ between the three agonists (Fig. 4b), but the maximal inhibition seen with corticosterone was less than that seen with either hydrocortisone or dexamethasone (Fig. 4c). The maximal response for the weak ligands (Fig. 4a) did not differ from that seen with vehicle, and therefore was not plotted (Fig. 4c).

The activity of the steroids was also compared using an end point assay, with cells harvested at 24 h (Fig. 5). The maximal inhibition was seen with dexamethasone, therefore set as 100%. The conventional GR agonists, hydrocortisone and corticosterone, were both effective, although corticosterone was markedly less active. Pregnenolone and 17-hydroxyprogesterone lacked a significant effect, but 21-hydroxyprogesterone significantly inhibited the IL-6-luc expression (Fig. 5).

To determine whether pregnenolone or 17-hydroxyprogesterone had the glucocorticoid receptor antagonist activity, their effects on the hydrocortisone response were measured. There was no antagonist effect observed (Fig. 6).

Discussion

A major physiological role for glucocorticoids is limiting the extent of inflammatory reaction (McMaster & Ray 2007). This activity is mediated by the activated glucocorticoid receptor translocating to the nucleus, and interacting with other transcription factors and transcriptional modulators (Chen et al. 2001, Stevens et al. 2003, Garside et al. 2004, O’Malley 2005, McMaster & Ray 2007). In vivo target cells are subject to multiple input stimuli, with varying durations of action. In order to dissect out specific pathways, a reductionist approach using cell culture has been successfully employed; however, the selection of single end points in cross-sectional analysis may give an incomplete picture of the full, natural response to stimulation. For this reason, we developed a genetically engineered IL-6 reporter cell line suitable for a continuous, non-destructive monitoring of promoter activity. Previous attempts to generate a cell-based bioassay for glucocorticoids have relied on transactivation by the GR (Vermeer et al. 2003), even though this mode of GR action is
not required for the most important physiological actions of glucocorticoids (Reichardt et al. 1998).

The reporter cells are robust and capable of survival in recording media for up to 10 days without detectable effects on cell viability. Over this period of time, the cells are sealed and the promoter activity monitored continuously as a result of exogenous luciferin present in the culture medium. This further enhances the responsiveness of the system by further shortening the half-life of the expressed luciferase (Takasuka et al. 1998). A preliminary analysis of the promoter response to glucocorticoid suggested a number of robust characteristics to the curve which could be measured, including the time to maximal inhibition, maximal inhibition and the overall integrated inhibition (area under the curve). We set out to measure these parameters in response to varying glucocorticoid concentration and also in response to different steroid structures.

The maximal inhibition–concentration response was the most sensitive to glucocorticoid, with the lowest measurable EC50. In contrast, the time to maximal inhibition showed a flat concentration–response curve, with no detectable effect of ligand dose. Interestingly, the EC50 for the overall inhibition–concentration response, that is the area under the dose–response curve, was consistently higher than that seen for maximal effect. An examination of the curves shows that the time to maximum effect is unaltered by concentration, but importantly, the overall duration of response increased with higher ligand concentration, and hence resulted in an increased area under the curve.

In vivo glucocorticoids act to oppose the effects of TNFα, and indeed using the reporter cell system dexamethasone abolished the TNF response of the IL-6

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**Figure 5** End point assay of inhibition of IL-6-luc after glucocorticoid precursor treatment. The cells were treated with hydrocortisone, corticosterone, dexamethasone, pregnenolone, 21-hydroxyprogesterone or 17-hydroxyprogesterone. A DMSO control was also included. After 24 h, the cells were harvested and an end point luciferase assay was performed. Inhibition was plotted as a percentage of the maximum inhibition induced by dexamethasone. Data from three independent experiments are presented as mean ± s.d. **P < 0.01 by comparison with vehicle treatment.

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**Figure 4** (a) The overall integrated inhibition of IL-6-luc by steroids. The cells were monitored for basal luciferase expression levels for 24 h, removed from photomultiplier tubes and treated with hydrocortisone, corticosterone, dexamethasone, pregnenolone, progesterone, 21-hydroxyprogesterone or 17-hydroxyprogesterone (1000 nM). A DMSO control was also included. The cells were then returned to PMTS and effects of the compounds on IL-6 promoter activity were monitored for 48 h. The overall integrated inhibition from fitted baseline was calculated for the 48 h time period following treatment. Results are displayed as percentage of maximum inhibition (hydrocortisone). Data from three independent experiments are presented as mean ± s.d. *P < 0.05 and **P < 0.01 by comparison with vehicle treatment. (b) The time to maximum inhibition of IL-6-luc by steroids. The time to maximum inhibition of IL-6-luc after 1000 nM steroid treatment. Data from three independent experiments are presented as mean ± s.d. Hc, hydrocortisone; Co, corticosterone; Dex, dexamethasone. (c) The maximum inhibition of IL-6-luc by glucocorticoid precursors. The level of maximum inhibition (largest deviation from baseline) was calculated as a percentage of the maximum inhibition induced by hydrocortisone. Data from three independent experiments are presented as mean ± s.d. Hc, hydrocortisone; Co, corticosterone; Dex, dexamethasone. *P < 0.05 compared with hydrocortisone.
In summary, we have developed and validated a new approach to measure steroid response \textit{in vivo}. This allows an accurate, sensitive and robust profiling of steroid activity in real time. This approach has revealed an unexpected complexity in the relationship between steroid structure and concentration on the different measurable parameters of response.

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