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Modulation of glucocorticoid receptor induction properties by cofactors in peripheral blood mononuclear cells

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Abstract

Glucocorticoids are widely used for their anti-inflammatory and immunosuppressive properties. Changing concentrations of transcriptional cofactors or chemicals in transiently transfected tissue culture cells are known to modify several properties of glucocorticoid receptor-regulated gene expression including total activity ($A_{\max}$), agonist steroid potency (EC_{50}), and the percent of full agonist activity for antisteroids (percent partial agonist activity). However, no reports exist for endogenous genes in primary human cells. Here we document that reduced concentrations of TIF2, a p160 coactivator, in peripheral blood mononuclear cells modulate these parameters for endogenous genes in a gene-selective manner, thus establishing the physiological relevance of this behavior.

Keywords

glucocorticoid hormone action; modulation of induction parameters; steroid potency vs. efficacy; antisteroids; gene-selective responses

1. Introduction

Lymphocytes and monocytes play an important role in human physiology. They are part of the defense in the immune system that protects the body against foreign objects and organisms. Glucocorticoids are widely used for their anti-inflammatory and immunosuppressive properties (1). Initially it was thought that these properties of glucocorticoid receptors (GRs) involve gene repression but it is now clear that GR-induced responses are also required (2,3). Glucocorticoids also kill lymphocytes and are used in treating lymphoproliferative diseases such as leukemias. However, many of the molecular details of GR action in lymphocytes, and with endogenous genes, remain obscure.

The current model of steroid hormone action, and glucocorticoid hormone action in particular, is based largely on studies in tissue culture cells and cell-free extracts. However, recent gene-specific and genome-wide studies indicate a wider range of responses than initially formulated, indicating the importance of examining aspects of the general model under more physiologically relevant settings (4,5). While the common approach is to concentrate on factors influencing the total amount of gene induction (and repression), or $A_{\max}$, it is becoming clear that additional parameters should be examined in order to comprehend the role of steroids

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during development, differentiation, and homeostasis. Two additional parameters of physiological significance are EC_{50} and percent partial agonist activity (6,7). The EC_{50} is the concentration of steroid required for half-maximal response and is a major determinant of the level of transcription of a steroid-regulated gene by circulating concentrations of agonist steroid. Furthermore, studies of both EC_{50} and A_{max} can yield mechanistic information not obtainable when observing just A_{max} (Ong et al., submitted). The percent partial agonist activity is the residual activity displayed by most antisteroids, expressed as percent of maximal activity of an agonist under the same conditions. Ideally, endocrine therapy with antisteroids blocks the action of endogenous agonist steroid on the gene of interest while retaining enough agonist activity with other target genes to minimally affect the overall constellation of regulated responses.

Changing the levels of various factors has been shown to modulate the A_{max} , EC_{50} , and percent partial agonist activity for induction and/or repression of synthetic and endogenous genes for most of the classical steroid receptors (6,7). However, these data are predominantly from tissue culture experiments. Very little evidence exists from primary cells or animals, mostly because few such studies have been reported. Increasing GR concentration by 60% shifts the dose-response curve for killing thymocytes to lower steroid concentrations by a factor of 10 (8). A mutant GR (I747M) causing familial glucocorticoid resistance has a 20–30 fold increase in EC_{50} due to reduced binding of coactivators (9). A common mutant ER (K303R) in a type of early premalignant breast lesions has increased affinity for TIF2 and AIB1 coactivators and displays a left-shift in the dose-response curve for cell growth (10). The syndrome of pituitary-specific thyroid resistance appears to result, at least in part, from mutations in thyroid receptor (TR) that reduce the affinity, and activity, of ligand-bound TR β 2 for coactivator but not corepressor (11).

The purpose of this study was to examine whether changes in transcription factor concentration can alter any parameters for GR induction of an endogenous gene in primary human cells. As our model system, we chose peripheral blood mononuclear cells (PBMCs), which are an easily obtained, extensively studied, and important target of glucocorticoids (12). We report that reduced concentrations of a p160 coactivator alter the EC_{50} or percent partial agonist activity of endogenous genes in a gene-selective manner, thus establishing the physiological relevance of these changes.

2. Materials and Methods

2.1. Isolation, freezing and thawing the PBMCs

PBMCs were isolated from human buffy-coat with Ficoll-PaqueTM Plus (Amersham), according to the manufacturer's instructions. Aliquots (1 ml) were cryopreserved at -80°C , and stored in liquid nitrogen, after resuspending at $5\text{--}10\times 10^7$ cells/ml in 80% RPMI 1640 medium, penicillin/streptomycin, 2 mM L-glutamine, 20% FBS and adding an equal volume of 40% RPMI 1640 medium, penicillin/streptomycin, 2 mM L-glutamine, 40% FBS, and 20% DMSO. Cells were rapidly thawed at 37°C and diluted into 9 ml of PBMC culture medium (90% RPMI 1640 medium with penicillin/streptomycin and 2 mM L-glutamine, 10% FBS). After centrifugation ($200\times g/10$ min/rt), the cells were resuspended in PBMC culture medium and cell number and viability were determined.

2.2. Reagents and antibodies

Dexamethasone (Dex) was purchased from Sigma. Dex-21-mesylate (Dex-Mes) was from Steraloids. Anti-TIF2 mouse monoclonal antibody (#610984; BD Biosciences) is commercially available. The GREtkLUC reporter is a synthetic plasmid in which a tandem repeat of the second glucocorticoid response element (GRE) of the rat tyrosine

aminotransferase gene is fused upstream of the thymidine kinase (tk) promoter driving the firefly luciferase (LUC) gene (13). pSG5/TIF2 (14) is from Heinrich Gronemeyer (IGBMC, Strasbourg, France). pSG5/human serum albumin (hSA) has been previously described (15).

2.3. Cell culture, transient transfection, and reporter analysis

Unstimulated PBMCs (2×10^6) were transfected with GREtkLUC reporter (4 μ g) and the internal control PGL4-renilla (1 μ g; Promega), without or with 0.5 μ g (or 1 μ g) pSG5/TIF2 or control pSG5/hSA, using Amaxa Human T-cell nucleofactor kit (VPA-1002) according to the manufacturer's instructions. Transfected cells were seeded into 12-well plates in 1ml PBMC culture medium at 2×10^6 cells per well, treated with different concentrations of Dex and Dex-Mes for 8 hr, lysed, and assayed for reporter gene activity using dual luciferase assay reagents according to the manufacturer's instructions (Promega, Madison, WI) on an EG&G Berthold luminometer (MicroLumat LB 96P). The data were normalized for total protein or *Renilla* luciferase activity and expressed as a percentage of the maximal response with Dex before being plotted $\pm$ S.E.M.

2.4. RNA extraction, reverse transcriptase PCR, and real-time PCR

RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Random-primed first-strand cDNA was prepared from 2 μ g of total RNA using SuperScript III reverse transcriptase (Invitrogen). The relative levels of target mRNAs were quantitated using SyberGreen and an ABI 7900HT real-time PCR system for glucocorticoid-induced leucine zipper (GILZ), GR, TIF2, CD163, and thrombospondin 1 (THBS1) using the below primers. The internal control, glyceraldehyde-3-phosphate dehydrogenase (primer 4310884E from ABI), was quantitated by Taqman.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
GILZ	AATGCGGCCACGGATG	GGACTTCACGTTTCAGTGGACA
CD163	TTCACCTGCACTGGGACTGAG	AGGACAGTGTTTGGGACTGG
THBS1	TTGTCTTTGGAACACACCA	CTGGACAGCTCATCACAGGA
TIF2	GGAAGGACCTGGTAAGAAGG	GCAACAAGAGTGCCATCAGA

2.5. RNAi in PBMCs

Small interfering RNA (siRNA) oligonucleotides for TIF2 and lamin (control) were purchased from QIAGEN. PBMCs (2×10^6) were transfected with siRNA oligos (100 pmol) using the Amaxa Human T-cell nucleofactor kit and seeded into 12-well plates in 1ml PBMC culture medium (2×10^6 cells/well). After 40 hr, cells were treated with different concentrations of Dex or Dex-Mes for 8 hr before being assayed for total proteins or mRNA levels as described above.

2.6. Statistical Analysis

Unless otherwise noted, the values of n independent experiments, were analyzed for statistical significance using InStat 2.03 (GraphPad Software, San Diego, CA). Best-fit curves (R^2 almost always were ≥ 0.95) following Michaelis-Menten kinetics were obtained for the dose-response experiments with KaleidaGraph (Synergy Software, Reading, PA).

3. Results and Discussion

3.1. Endogenous GR induction of an exogenous reporter

The majority of cells present in PBMCs contain endogenous GRs. We therefore asked if these receptors are capable of inducing the exogenous luciferase reporter plasmid GREtkLUC, which

is well-known to display altered $A_{\max}$, EC_{50} , and percent partial agonist activity with varying levels of receptor and other transcription factors (6,7). We used unpurified and unstimulated PBMCs in an effort to minimize experimental manipulations, thereby yielding cells that are as close as possible to those in the human circulatory system. Marginal Dex inducibility of transfected GREtkLUC was observed in fresh, unstimulated PBMCs, apparently due to very inefficient transfection of reporter DNA (data not shown). However, cryopreserving and thawing the PBMCs, followed by the addition of steroid immediately after Amaxa transfection caused the fold increase in gene induction to increase by a factor of two and gave the predicted Michaelis-Menten dose-response curve (Fig. 1), which goes from 10% to 90% response over an 81-fold concentration of steroid (Ong et al., submitted). Additional TIF2 coactivator is well-documented to dramatically increase the $A_{\max}$ and decrease the EC_{50} of GREtkLUC and other responsive genes (7,16). Unfortunately, this treatment of PBMCs is comparatively ineffective (Fig. 1), apparently due to minimal expression of transiently transfected TIF2 plasmid above the relatively high levels of endogenous TIF2 in PBMCs (data not shown and Fig. 2A below).

3.2. Effects of TIF2 siRNA

We next attempted to lower the high levels of endogenous TIF2. Transient transfection of TIF2 siRNA for 48 hr successfully reduced endogenous TIF2 protein (Fig. 2A) and mRNA by at least 50% (Fig. 2B). Little if any decrease was observed 8 or 24 hr after adding TIF2 siRNA (data not shown). There was no induction of exogenous GREtkLUC, though, after 48 hr so we examined the endogenous genes GILZ, CD163, and THBS1, which are highly induced by Dex in PBMCs (4). In control cells treated with Lamin siRNA, the fold induction of GILZ, CD163, and THBS1 by Dex are similar (15.5 ± 2.2 , S.E.M., $n = 19$) but the EC_{50} values range from 3 to 16 nM and are statistically different ($P = 0.0026$ by Kruskal-Wallis non-parametric ANOVA). The effects of added TIF2 siRNA are also gene-selective. Representative dose-response curves $\pm$ TIF2 siRNA are shown in Fig. 2C–E. The average values of the fold induction (approximately equal to $A_{\max}$ because the uninduced levels are very similar), EC_{50} , and percent partial agonist activity of Dex-Mes (% DM) of gene induction from 6–7 independent experiments are given in Fig. 2F. The EC_{50} is significantly increased by TIF2 siRNA for THBS1, is not quite significantly increased for CD163 ($P = 0.063$), and is unchanged for GILZ. Conversely, the percent partial agonist activity is significantly decreased by TIF2 siRNA for GILZ but not CD163 or THBS1. The decrease in $A_{\max}$ is not quite significant for GILZ ($P = 0.053$) while no change is seen for CD163 or THBS1. Thus the effects of decreasing the concentration of endogenous TIF2 on the fold induction, EC_{50} , and percent partial agonist activity of gene induction are gene- and function-selective.

3.3. How TIF2 siRNA affects the EC_{50} or $A_{\max}$

We recently developed a theoretical mathematical model of steroid hormone action that predicts the independent effects on various transcription parameters seen in Fig. 2 and makes mechanistic predictions based on the behavior of $A_{\max}$ and EC_{50} (Ong et al., submitted). Specifically, this model says that the increase in EC_{50} of THBS1 induction by TIF2 siRNA with no change in $A_{\max}$ is indicative of TIF2 siRNA acting as a linear or competitive inhibitor, although the location of the inhibition is not uniquely specified. The most likely site of action is a pathway that feeds into the GR induction pathway, i.e., the presence of the co-activator TIF2. It should be noted that this mechanism is not the exact reverse of what is usually observed when TIF2 is elevated, i.e., increase in $A_{\max}$ and decrease in EC_{50} (17–19). The reason for the non-mirror symmetry when TIF2 levels are increased vs. decreased is thought to be that the endogenous level of TIF2 is at the cusp of two different mechanistic pathways. Thus raising or lowering the TIF2 concentration tilts the process to follow different paths. We cannot yet test this prediction experimentally because of our above-noted inability of transiently transfected TIF2 plasmid to significantly increase the levels of TIF2 protein.

3.4. Relevance of differential responses

This study is the first documentation that altering the level of a specific endogenous transcription factor can change some GR induction properties of endogenous genes in primary human cells. This supports the hypothesis that the modulation of GR induction properties is a relevant feature of human physiology and can provide a viable mechanism for the differential control of gene expression during development, differentiation, and homeostasis (6,7). The fact that the responses are not general but affect only selected parameters of specific genes suggests that different pathways and/or factors exist for the selective control of $A_{\max}$, EC_{50} , and percent partial agonist activity, which is consistent with the many in vitro findings with tissue culture cells (6,7) and a recent theoretical study (Ong et al., submitted). This selectivity is relevant because it provides additional diversity for the differential control of gene expression. The data further suggest that targeting specific cofactors during endocrine treatments, such as immunosuppression by glucocorticoids and antiglucocorticoid treatment of glucocorticoid excess, can uniquely modify selected gene induction parameters, thereby attenuating GR actions with a greatly reduced population of genes while retaining most activity with the majority of responsive genes.

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Fig. 1. Endogenous GR induction of an exogenous reporter in PBMCs. (A) Transfected GREtkLUC reporter is induced by Dex in PBMCs in a dose-dependent manner. (B) Data of A are replotted to give Michaelis-Menten dose-response curves with Dex for increasing amounts of transiently transfected TIF2 plasmid.

Fig. 2.

Effects of TIF2 siRNA on GR-regulated endogenous genes are gene-specific. Transient transfection of TIF2 siRNA for 48 hr reduces endogenous TIF2 protein (A: with EtOH; Western blot) and mRNA (B: with EtOH, Dex, or DM; real-time PCR) by at least 50% relative to Lamin siRNA. (C-E) Representative dose-response curves for Dex induction of endogenous GILZ, CD163 and THBS1 in PBMCs with or without transfected TIF2 siRNA are shown. (F) The average values of fold induction (approximately equal to $A_{\max}$ because uninduced levels are very similar), EC₅₀, and percent partial agonist (% DM) activity of gene induction from 6–7 independent experiments are plotted. * $P=0.038$, ** $P=0.0008$.